A study of saponins in legumes of importance to both human and animal nutrition

Thesis

A STUDY OF SAPONINS IN LEGUMES OF IMPORTANCE TO BOTH HUMAN AND ANIMAL NUTRITION

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Abstract

This work describes the analysis and isolation of saponins using a range of chromatographic techniques, the development of a quantitative HPLC method for the determination of saponins as they occur in seed, and the study of various factors which affect both the saponin content and composition in different legume seeds.

Seed from 6 cultivars of lupin were grown in 4 regions of Australia, with up to 15 plots sampled within each region. Saponin contents ranged from 0 to 740 mg kg^{-1}. Soyasaponin I, soyasaponin VI (the 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one conjugate of soyasaponin I) and a new saponin were found in the lupin seed. The changes observed in saponin content were dependent on cultivar, followed by the site and then the region where grown.

The saponin content of 20 cultivars of lentils grown in Spain ranged from 654 to 1269 mg kg^{-1}. Soyasaponins I and VI were present in all the cultivars. The saponin content depended on both seed size and testa colour.

The use of a mild extraction technique and an improved quantitative HPLC method showed soyasaponin VI to be the only saponin present in lupin seed whereas soyasaponin I was derived from soyasaponin VI when harsher extraction conditions were used.

The HPLC method was used to study the effect of soaking, cooking and germination on the saponin content and composition of two cultivars of both chickpeas and lentils. Soyasaponin VI was the only saponin detected in the unprocessed, soaked and germinated seed. Neither soaking nor germination modified the saponin content or composition of chickpeas and lentils. Soyasaponin VI was partially degraded during cooking into soyasaponin I, and both leached into the cooking solution. An overall loss of saponin content was found for lentil but none for chickpea.

Abbreviations

ara	L-arabinose
CI	chemical ionization
CMC	critical micelle concentration
CTLC	centrifugal thin-layer chromatography
DCCC	droplet counter current chromatography
DDMP	2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one
EI	electron ionization
ELISA	enzyme linked immunosorbent assay
ES	electrospray
ESR	electron spin resonance
FAB	fast atom bombardment
FD	field desorption
gal	D-galactose
GC	gas chromatography
glc	D-glucose
glcUA	D-glucuronic acid
HPCE	high-performance capillary electrophoresis
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin-layer chromatography
IR	infrared spectroscopy
ISCOM	immuno stimulating complex
LSIMS	liquid secondary ion mass spectrometry
man	D-mannose
M_r	relative molecular mass

MS	mass spectrometry
NMR	nuclear magnetic resonance spectroscopy
P	statistical probability
PAD	pulsed amperometric detection
RDA	Retro Diels-Alder
rham	L-rhamnose
SOD	superoxide dismutase
SSI	soyasaponin I
SSVI	soyasaponin VI
SSX	novel saponin
TLC	thin-layer chromatography
TMS	trimethylsilyl
UV	ultraviolet spectroscopy
xyl	D-xylose

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I Introduction

1.1 LEGUMES

Legumes can be defined as the fruit of the large family Leguminosae, but the name is used more commonly for the plants themselves. Although the family comprises 700 genera, only about 15 of them contain significant crop plants.

The word "legume" is derived from the latin "legumen" which means seeds harvested in pods, although an alternative term for edible seeds of leguminous plants is "pulse" from Latin "puls", meaning pottage. The current literature sometimes refers to leguminous seeds as grain legumes, whilst the term 'food legumes' is used to cover both the immature pods and seeds as well as mature dry seeds, which are consumed by human beings or domestic animals. Of the various legumes cultivated, soyabeans, groundnuts, dry beans, peas, broad beans, chickpeas and lentils are the major food legumes grown in all continents of the world[1].

1.1.1 Importance of grain legumes

Food legumes offer a relatively inexpensive source of protein which is valuable for developing countries. The seeds of these plants are the part most commonly eaten and most of them can be easily stored for future use.

Legume seeds contribute less than 10% of man's daily per capita energy and protein supply, while about 50% comes from cereal grains, in spite of the fact that legume seeds, in some respects, are nutritionally superior to cereals. In particular they contain at least twice as much protein (average 20-26 per cent) and also a large amount of carbohydrate, most of which is digestible starch. In general, they are rich in the essential amino acid lysine, but poor in the sulphur-containing amino acids. Since the reverse is true for cereals, a combination of the two can approach the high nutritive value of animal protein. Grain legumes are considerably richer in calcium than most cereals. They are also

rich in iron, thiamin, riboflavin, and nicotinic acid when compared to cereals.

However, in spite of their obvious nutritional advantages, legumes seeds are known to contain several antinutritional factors, such as trypsin and chymotrypsin inhibitors, phytates, lectins, polyphenols, flatulence factors, cyanogenic compounds, lathyrogens, estrogens, goiterogens, saponins, antivitamins, and allergens[2].

Traditional methods of processing legumes are often used to produce wholesome edible products with a reduced level of the toxic compounds, although the degree of elimination of toxic constituents will depend on the type of legume and processing conditions. Heat treatment is well established as a method to destroy proteinaceous antinutrients, such as protease inhibitors and lectins, but this treatment may also destroy some of the amino acids and vitamins. To maintain the nutritional value of food subjected to heat treatment, it is necessary to make certain that the temperature and processing time reach, but do not exceed, the optimum conditions required to eliminate the effect of inhibitors without altering basic nutrients. Proteins in legumes are known to interact with lipids, tannins, phytates, flavour compounds, and pigments[3] and these interactions will occur when legumes are processed, which in turn will decrease the bioavailability of the proteins. Similarly, tannins and phytates interact with minerals and vitamins, resulting in a decrease in their bioavailability[4]. Thus, bioavailability of nutrients depends not only on the content of the nutrients in the seed, but also on the interaction of nutrients with other compounds under various processing conditions.

In addition to their value as a foodstuff, legumes are also important in agronomic terms because of their ability to produce nitrogen and increase the overall fertility of the soil, thus partially replacing the use of expensive nitrogenous fertilizers. This arises as a consequence of a fascinating interaction between legume roots and *Rhizobium* bacteria which fix nitrogen. It results in the formation of a highly developed organ within which the bacteria grow and convert nitrogen gas from the atmosphere into ammonia, which can be assimilated by the plant.

The dominant food legumes vary from country to country, and from region to

region, but most of them can be grown under a reasonably wide range of ecological conditions and many can be grown satisfactorily on poor soils without the application of artificial fertilizers.

1.1.2 Lupin (*Lupinus*)

Lupin is attracting worldwide attention not only for its ability to improve soil quality but also for its potential as a grain legume to provide high-quality protein, fat and fibre.

1.1.2.1 *Botany*

The cultivated grain lupins are tall-growing, flowering plants. The flowers are borne in large, showy terminal racemes and can be white, pink, blue or purple in colour (Figure 1.1). The pods are flattened, mostly constricted and usually contain 3 to 6 seeds. Seeds of lupin varieties have shapes that range from round and flat to nearly spherical. Bitter tasting varieties tend to have mottled, dark grey to dark brown seeds whilst the sweet tasting varieties tend to be white or cream.

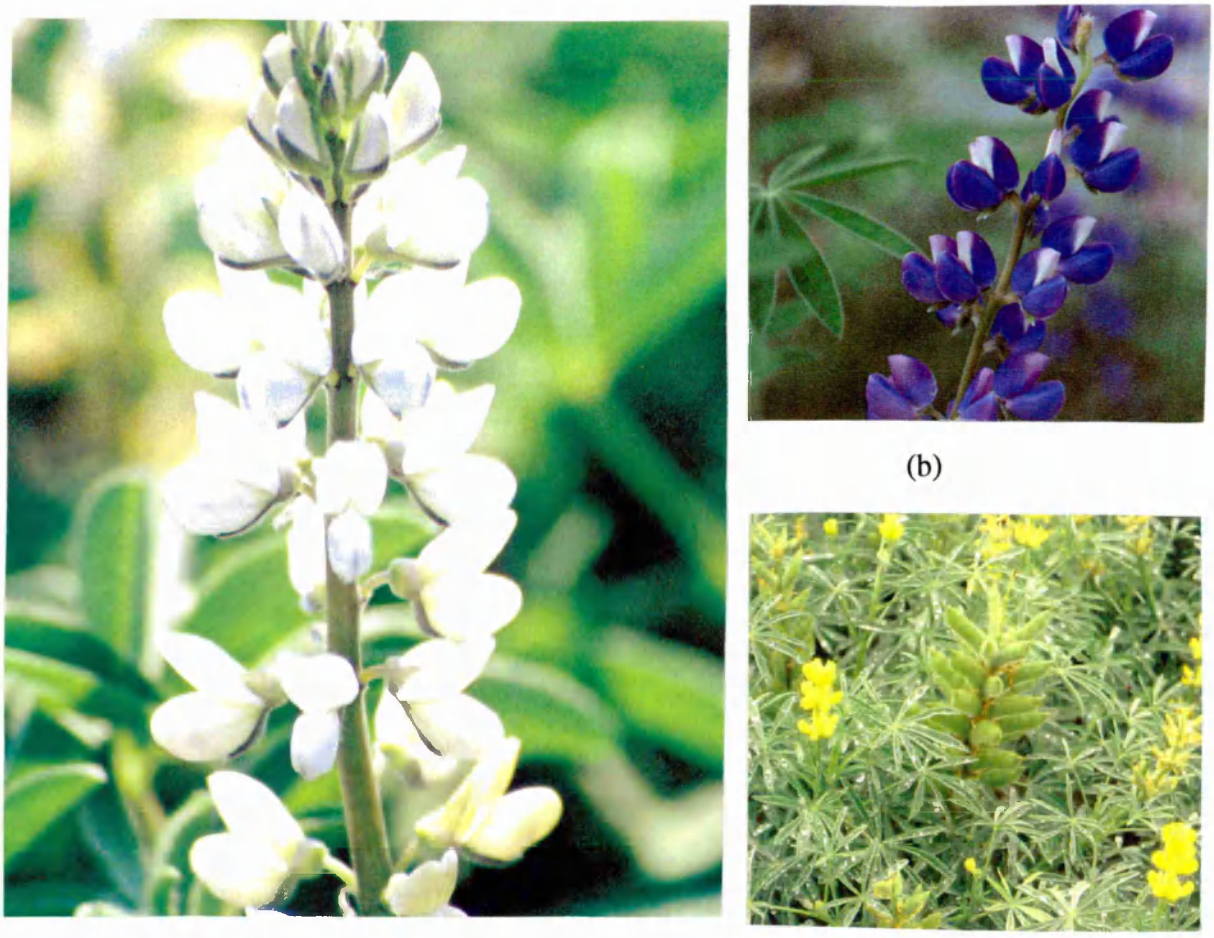
The differences between the species of *Lupinus* are rather poorly marked, identification is sometimes difficult, and some botanical names have become misapplied, but *Lupinus albus*, *L. angustifolius*, *L. luteus* and *L. mutabilis* are of importance as grain legumes.

1.1.2.2 *Origin and distribution*

In times past, the Egyptians, Greeks and Romans used *Lupinus albus* as a grain and as a soil enricher. Native South Americans consumed lupins after soaking and cooking which helped to remove the bitter taste caused by alkaloids. The Andean highland civilizations cultivated *L. mutabilis* extensively and toasted or cooked the grains to make them edible[5-8].

In modern times, *L. albus* was introduced to Germany during the 18th century with results poorer than expected, but cultivation of lupin in the sandy Baltic coastal plains prospered, although it was used predominantly as an animal feed and soil enricher. The appearance of lupin-associated toxicity in sheep led to a diminished interest in their cultivation up to World War I but, during the war, breeders in Germany developed "sweet" (low-alkaloid) varieties of lupins that allowed this crop to be more desirable for livestock feeding[9].

Nowadays, commercial lupin cultivars are grown over a wide range of latitudes, but only within a restricted range of growing-season temperatures. The crop is produced as a summer annual in the cool temperate climates of Northern Europe and New Zealand and as a winter annual in subtropical climates such as the South Eastern United States[10].



(a) (b) (c)

FIGURE 1.1. (a) *Lupinus albus*, (b) *Lupinus mutabilis*, (c) *Lupinus luteus*.

1.1.2.3 *Chemical composition*

Different cultivation conditions can modify the chemical composition of the lupin seed whilst larger and fuller seeds have proportionately more protein and less crude fibre[8].

The protein content of whole lupin seeds is similar to that of soya beans. *L. angustifolius* contains approximately 31% protein on a dry weight basis while *L. albus*, *L. luteus* and *L. mutabilis* have up to 37%, 42% and 43% protein respectively[11-13]. These values are nearly double those of some legumes commonly used as human foods, such as chickpeas, peas and several beans.

Lupin protein, as is true for all legumes, has a low content of sulphur-containing amino acids (methionine and cystine) and is also relatively low in valine and tryptophan although high in lysine, glutamic acid, aspartic acid and arginine[8]. This pattern is typical for a legume protein and complements the pattern in cereal grains in terms of their high lysine and methionine contents[14,15].

The oil content of lupin is lower than that of soya, with *L. albus* containing 11% lipids on a dry weight basis while other species have less than 6%. However, *L. mutabilis* may have up to 20%, which is similar to soya and 2-3 times the oil content of other legumes[8,11].

Although the oil yield, as a proportion of dry seed, is somewhat low in comparison with soyabeans, the oil is readily extractable and can be refined by conventional processes to yield a pale bland edible oil in good yield. Owing to the presence of linolenic acid, lupin seed oil, like soyabean oil, has limited stability in ambient storage conditions but its quality compares favourably with that of soyabean oil or rapeseed oil[16].

The crude fibre content for whole seeds varies from 11 to 17% on a dry weight basis, higher than that of soya bean, and over 80% is present in the hull. Even among legumes, lupin has a particularly high dietary fibre content[7] which consists mostly of a non-starch, non-cellulosic polysaccharide which has a rhamno-galacturonan backbone,

with galactose and arabinose present as side-chains. Small amounts of cellulose and hemicellulose are also present[13].

Generally, the seeds of legumes contain anti-nutritive substances which can considerably reduce their value. For lupins, the major hindrance to their wide use in human and animal nutrition is the quinolizidine alkaloids which are toxic and bitter, although their levels have been reduced by breeding programmes and have resulted in the so-called "sweet" varieties[9].

The level of alkaloids in modern varieties of sweet lupin is considered safe and other antinutritional factors, such as those found in soya, are generally at lower levels in the lupin. Petterson *et al.*[17] found phytates, trypsin inhibitors, tannins, phytohaemoglutinins and oligosaccharides to be present in lupin seeds, but their concentrations were lower than in other legume species. However, saponins, although still considered as antinutritional factors but now also thought to have some beneficial properties, might be present in lupin and interfere with the nutritional use and acceptance of lupin in the human diet.

1.1.2.4 *Main and potential use*

Ground lupin seed is utilized as a source of protein in concentrates for cattle, sheep, pigs and poultry. However, the modern sweet lupin seed, with its low levels of alkaloids, offers a broad range of possibilities for use in human food systems[18]. Such applications include its use as an alternative to soya bean in the preparation of tempeh[19], miso, 'milk' products[6,8], or its use as a nutritional and functional food ingredient in the form of flours[20], protein isolates and concentrates[21] and particularly in fibre preparations[7,22].

1.1.3 Lentil (*Lens culinaris* Medik.)

Lentils have made a significant contribution to the human diet since ancient times. As a

pulse crop, lentils are a very important component of tropical agriculture and provide a highly nutritious and protein-rich food.

1.1.3.1 *Botany*

The lentil is a much-branched, sub-erect plant. The flowers may be solitary, or in racemes of 2-4 flowers, and may be white, pink, red or violet in colour, according to the cultivar (Figure 1.2). The seed-pods are smooth, compressed and contain two smooth lens-shaped seeds, which show considerable variation in size and colour.

There are two subspecies of lentil, morphologically well defined, each with a definite geographic area: ssp. *macrosperma*, with large, flattened seeds, and ssp. *microsperma*, with small or medium-sized seeds. The latter is more polymorphous and within it, six narrower geographic groups, or varieties, may be designated.

While the subspecies *macrosperma* includes the so-called Chilean types of lentil, the subspecies *microsperma* includes the small-seeded Persian lentil[10].

FIGURE 1.2. *Lens culinaris.*

1.1.3.2 *Origin and distribution*

Lentils are one of the oldest of the grain legumes and are thought to have originated in Asia Minor, but quickly spread to Egypt, central and southern Europe, the Mediterranean basin, Ethiopia, Afghanistan, northern India and Pakistan. They were successfully introduced into the New World and are now grown and consumed everywhere in the world[10,23].

1.1.3.3 *Chemical composition*

The proximate composition of lentil has been widely reported in the literature[24]. However, the data are not always comparable due to differences such as genotypes, environments and methods of analysis.

Although low in methionine and cystine, lentil is an excellent source of protein and amino acids to complement cereal protein, particularly wheat, with which it is most often eaten in the developing countries. Lentils contain protein in the range 22 to 31% on a dry weight basis, which is a comparable level to that present in most other legumes[23].

Lentils contain about 69% carbohydrates on a dry weight basis, most of which is present in the form of starch. Reddy *et al.*[25] reported starch and dietary fibre contents of lentils in the range of 35 to 53% and 4 to 5% respectively. The amylose content of lentil starch was in the range of 21 to 46%. The dietary fibre consists of hemicelluloses, cellulose, and lignin. As most of the dietary fibre is present in the seed coat, the dehulled product, *dhal* contains very low amounts of fibre.

The lipid content of lentil seed is in the range of <1 to 4% (dry weight)[23], comprising mainly oleic and linoleic acids[26].

Lentil seeds contain 2 to 4% minerals[24] with phosphorus and iron concentrated in the cotyledon. The seed coat, which represents only 8% of the whole seed, contains almost 40% of the total calcium. Lentils are relatively good sources of potassium, iron, zinc, and magnesium, and also contain significant amounts of the B vitamins, thiamin and

riboflavin[23].

Although lentils are considered to be one of the most nutritious pulses, they also contain several antinutritional factors which could limit their consumption[23].

1.1.3.4 *Main use*

Lentils are a nutritious foodstuff and are used mainly for human consumption. Methods of preparing lentils vary widely among different ethnic groups and are mainly used as an ingredient in soups or in the form of *dhal*. In some parts of India, the whole seeds are often salted and fried. Flour prepared from the ground seeds can also be used as a mixture with cereal flours for cake- or bread- making and in the preparation of baby foods[10].

1.1.4 Chickpea (*Cicer arietinum*)

Chickpea is one of the oldest and most widely grown legumes in the world. The nutritional quality of chickpea proteins is acknowledged as the highest of all the pulses[27].

1.1.4.1 *Botany*

The chickpea is a small, herbaceous, annual shrub, showing considerable variation in form. Some types are semi-erect with a main stem and only a few branches, while others are semi-spreading with profuse branching. The flowers are usually solitary and formed in the axils of the leaves on a jointed peduncle; they are normally pinkish, purplish, red fading into blue, or white, or occasionally greenish-white or blue (Figure 1.3). The seed-pods are oblong and generally contain one or two relatively large seeds, which vary in colour.

Distinct geographical forms of chickpeas exist and four races have been recognized, namely *oriental*, *asiaticum*, *mediterranean* and *eurasiaticum*; in addition, five

subraces within the race *oriental* and four within the race *asiaticum*, are also recognized[10]. Based on seed colour and geographic distribution, chickpea is grouped into two types: *desi* (Indian origin) and *kabuli* (Mediterranean and Middle Eastern origin). *Kabuli* cultivars are white to cream coloured and used almost exclusively for cooking whole as a vegetable. The seeds of *desi* cultivars are wrinkled with a brown, light brown, fawn, yellow, orange, black, or green colour. These cultivars are normally dehusked to obtain *dhal* which is directly cooked or milled to a flour [28].

FIGURE 1.3. *Cicer arietinum.*

1.1.4.2 *Origin and distribution*

The origin of the chickpea is a little obscure. Some authorities consider that the species did not exist in the wild, but was developed artificially by man, and that there were two centres of origin[10]. India, Central Asia, Near Eastern, and Mediterranean Centres are primary and the Ethiopian centre secondary sources of origin for chickpea. Chickpea is an

important crop of the Indian subcontinent, the Mediterranean region, the Middle East, Ethiopia and several other African countries, and some European countries. It was introduced successfully into the New World, and has become an important crop in Mexico, Argentina and Chile. More recently, it has been introduced into Australia, although cultivation there is not yet of commercial importance[28].

1.1.4.3 *Chemical composition*

The chemical composition of chickpea seed components has been reported[29]. The cotyledons contain about 96% of the proteins, 94% of the fat, 81% of the ash, 88% of the carbohydrates, 94% of the phosphorus, and 70% of the iron of the whole seed. Although the embryo is rich in proteins, fat, and minerals, its contribution is small on a total seed weight basis. The seed coat contains most of the non-digestible carbohydrates and a relatively higher proportion of calcium, and so the cotyledons are the major source of nutrients.

The crude protein content of chickpea seeds ranges from 12 to 31%, with an average of 22% on a dry weight basis, and that of *dhal* ranges from 21 to 31%, with an average of 26%[30]. Chickpea protein has a low content of sulphur-containing amino acids and is also relatively low in valine, threonine and tryptophan[31].

Chickpea seeds contain 52 to 71% (dry weight) of total carbohydrates of which the major proportion is made up of starch[30].

The total lipid content in the chickpea ranges from 3% to 7% on a dry weight basis. Linoleic and oleic acids in the unsaturated fatty acid group and palmitic acid in the saturated fatty acid group are the principal fatty acids[32].

The chickpea is a good source of dietary minerals, such as calcium, phosphorus, magnesium, iron and potassium[33].

The information on the vitamin composition of the chickpea is scanty. The chickpea contains a considerable amount of ascorbic acid[34].

Although chickpeas are an excellent source of protein, carbohydrates and

minerals, several antinutritional factors are also found in biologically significant amounts in the raw seeds of these leguminous crops[28].

1.1.4.4 *Main use*

In the principal producing areas chickpeas are used in various forms, mainly for human consumption. In India about 75 per cent of production is consumed as *dhal*. Elsewhere the whole mature seeds are used in a wide range of dishes, often after they have been soaked and boiled. In addition, the seeds may be parched, or roasted in hot pans and eaten similarly to roasted groundnuts, or used as an ingredient of various sweetmeats[10].

1.2 SAPONINS

Saponins are a group of naturally occurring compounds which form stable, soapy foams in aqueous solutions (the term saponin is derived from Latin *saponins* which means soap). They constitute a complex and chemically diverse group of compounds, mainly of plant origin[35-37] but also occurring in a number of marine animals[38,39].

Long before the first saponin was identified over 100 years ago[40], people from cultures as diverse as Medieval European and Australian Aboriginal[41] had used green twigs from certain plants to poison and harvest fish from water holes and ponds. Saponins are characterized by their toxicity against molluscs and other invertebrates and against aquatic vertebrates such as fish and tadpoles[35]. From the biological standpoint, saponins possess a diversity of other properties, some of which are deleterious but many of which are beneficial[35-37]. For this reason, there has been great interest, especially in the Orient, in the chemical characterization of the saponins responsible for the effects ascribed to plant drugs and folk medicines.

1.2.1 Nature and occurrence

Whilst all saponins contain an aglycone (sapogenol, sapogenin) linked to one or more sugars or oligosaccharide moieties, they fall naturally into two groups depending upon whether the aglycone is triterpenoid or steroidal. Almost all the saponins in food and forage plants are of the triterpene class.

Saponins are present in many hundreds of plant species, but only a few of these are used as food by man or domestic animals[35,36]. Table 1.1 includes a list of the main food plants used for human or animal consumption which contain significant levels of saponins.

TABLE 1.1. Saponins in plants used as foods and feedingstuffs.

• *Plants used as human foods*	• *Plants used as animal feedingstuffs*	• *Plants used as flavourings, health foods, tonics, etc.*
Soya	Alfalfa	Fenugreek
Beans (*Phaseolus* species)	Forage and clover crops	Liquorice
Other beans and peas	Sunflower	Nutmeg
Oats	Horse chestnut	*Quillaja*
Allium species	Guar	*Saponaria*
Asparagus	Lupin	*Yucca*
Tea		*Gypsophila*
Peanut		Herbs
Spinach		Guarana
Cucurbits		*Madhuca*
Sugar beet		Ginseng
Yam		
Quinoa		

1.2.2 Biosynthesis

Heftmann[42,43] published a detailed account of the biosynthesis of steroidal saponins and glycoalkaloids. The fundamental consideration is Ruzicka's "biogenetic isoprene rule". The fundamental proposition of this rule was that certain key acyclic terpenes, either

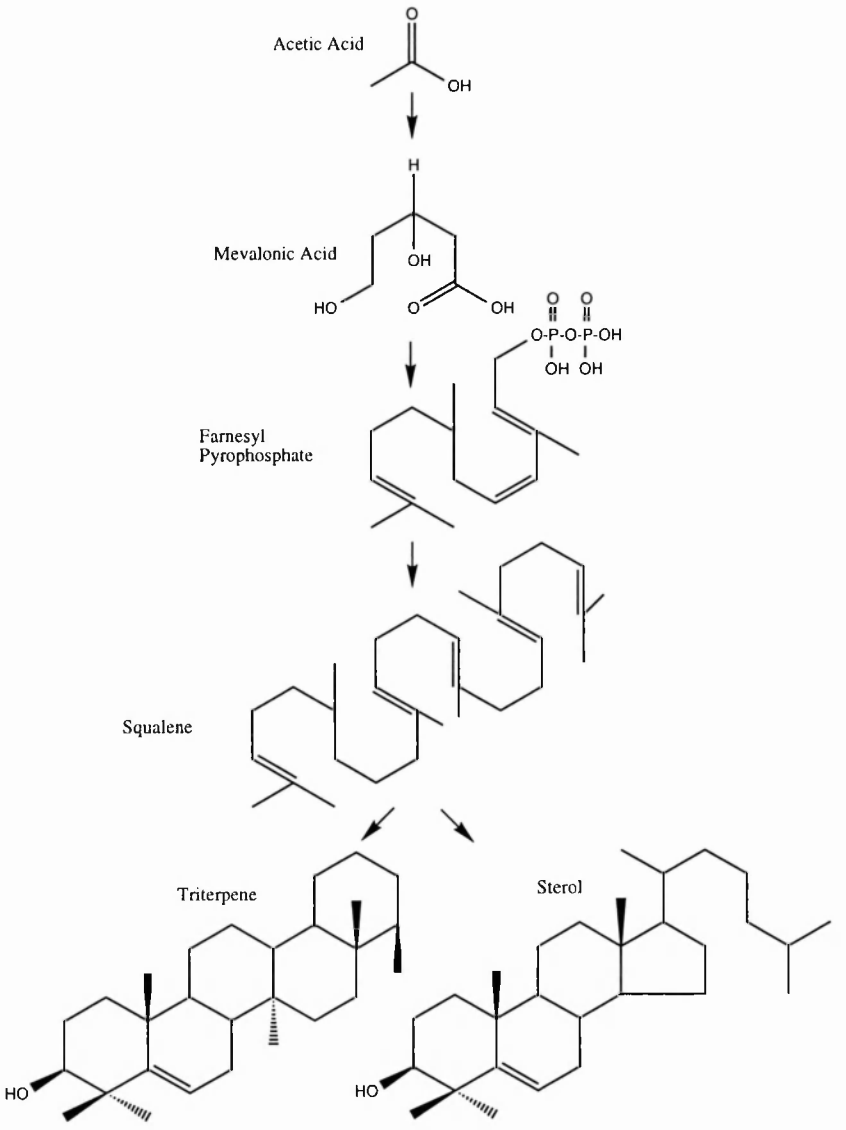


FIGURE 1.4. Biosynthesis of triterpenes and sterols.

known or (then) hypothetical, could "condense" to yield mono, sesqui-, di- and triterpenes. In the case of animal and plant triterpenes and sterols, the key acyclic precursor was considered to be squalene (Figure 1.4). Most saponins have an *O*-substituent at C_3 which has been shown to result from the intermediate 2,3-epoxysqualene.

Several experiments using different isotopic labelling approaches have shown that saponin biosynthesis is most active in the tissues most vulnerable to mould attack or insect predation[35].

1.2.3 Chemical structures

The complexity of saponin structure (and thereby the diversity of biological activities) has its basis not only in the variability of aglycone structure and position(s) of attachment of the glycosidic moieties, but also in the nature of the latter. Aglycones are generally linked to D-galactose (gal), L-arabinose (ara), L-rhamnose (rham), D-glucose (glc), D-xylose (xyl), D-mannose (man), and D-glucuronic acid (glcUA) some of which may be acetylated; chain lengths of 2-5 saccharide units are most frequent. The oligosaccharide chains are generally linear, although branching is not uncommon[37].

Given the complexity of these molecules within both the (oligo)saccharide and aglycone moieties there are no systematic rules for nomenclature. Consequently many saponins are named in a trivial manner which serves to specify the botanical origins of the saponins. Whilst such trivial nomenclature has obvious advantages, it may lead to confusion and has the potential for duplication. This is particularly the case with the soyasaponins.

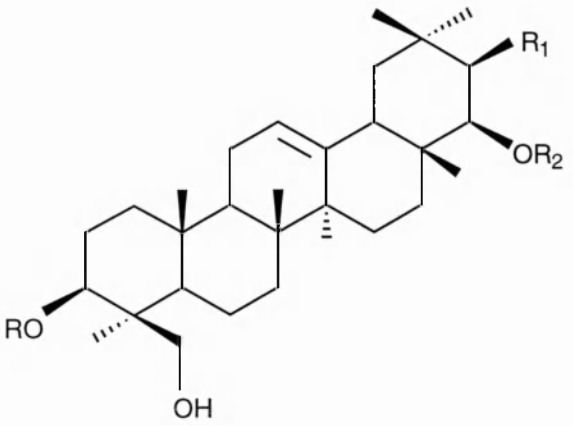


FIGURE 1.5. Structures of group A and B saponins.

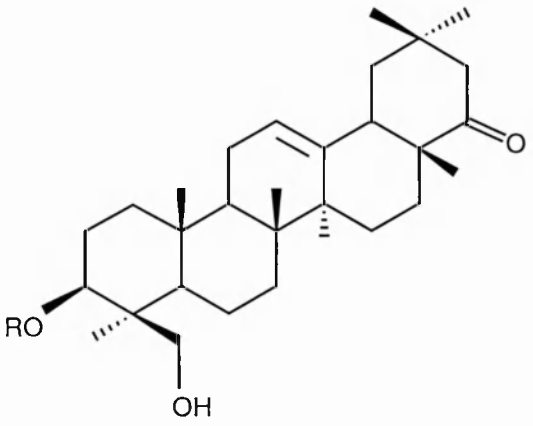


FIGURE 1.6. Structures of group E saponins.

R: -H *R*$_1$: -H *R*$_2$: -H Soyasapogenol B

R: -H *R*$_1$: -OH *R*$_2$: -H Soyasapogenol A

R: -H Soyasapogenol E

Name		*R*	*R*$_1$	*R*$_2$
Kitagawa	*Okubo*			
	A group			
acetylA4	Aa	Glc(1-2)gal(1-2)glcUA-	-OH	2,3,4-tri Ac.xyl(1-3)ara-
acetylA1	Ab	Glc(1-2)gal(1-2)glcUA-	-OH	2,3,4,6-tetra Ac.glc(1-3)ara-
----	Ac	Rham(1-2)gal(1-2)glcUA-	-OH	2,3,4,6-tetra Ac.glc(1-3)ara
----	Ad	Glc(1-2)ara(1-2)glcUA-	-OH	2,3,4,6-tetra Ac.glc(1-3)ara-
acetylA5	Ae	Gal(1-2)glcUA-	-OH	2,3,4-tri Ac.xyl(1-2)ara-
acetylA2	Af	Gal(1-2)glcUA-	-OH	2,3,4,6-tetra Ac.glc(1-3)ara-
acetylA6	Ag	Ara(1-2)glcUA-	-OH	2,3,4-tri Ac.xyl(1-2)ara-
acetylA3	Ah	Ara(1-2)glcUA-	-OH	2,3,4,6-tetra Ac.glc(1-3)ara-
A4	deacetyl Aa	Glc(1-2)gal(1-2)glcUA-	-OH	xyl(1-3)ara-
A1	deacetyl Ab	Glc(1-2)gal(1-2)glcUA-	-OH	glc(1-3)ara-
A5	deacetyl Ae	Gal(1-2)glcUA-	-OH	xyl(1-2)ara-
A2	deacetyl Af	Gal(1-2)glcUA-	-OH	glc(1-3)ara-
----	----	Rham(1-2)gal(1-2)glcUA-	-OH	-H*
A6	deacetyl Ag	Ara(1-2)glcUA-	-OH	xyl(1-2)ara-
A3	deacetyl Ah	Ara(1-2)glcUA-	-OH	glc(1-3)ara-
	B group			
V	Ba	Glc(1-2)gal(1-2)glcUA-	-H	-H
I	Bb	Rham(1-2)gal(1-2)glcUA-	-H	-H
II	Bc	Rham(1-2)ara(1-2)glcUA-	-H	-H
III	Bb'	Gal(1-2)glcUA-	-H	-H
IV	Bc'	Ara(1-2)glcUA-	-H	-H
	E group			
	Bd	Glc(1-2)gal(1-2)glcUA-		
	Be	Rham(1-2)gal(1-2)glcUA-		

* Isolated by Curl *et al.* (1988) and given the name soyasaponin A3

1.2.3.1 *Saponins in plants used for human consumption*

1.2.3.1.a Legumes

Soyasaponins, which are the most thoroughly investigated of the saponins occurring in food plants, may be subdivided into three groups, (A, B, and E respectively)[44-46], depending upon whether the aglycone is soyasapogenol A, B or E. The structures of these soyasapogenols together with those of typical soyasaponins are shown in Figures 1.5 and 1.6. If sapogenol A contains two ether-linked sugar chains (attached to positions 3 and 22) it is termed a *bis-desmoside*; in soyabeans, soyasapogenol B contains sugars attached to position 3 alone and is thus a *mono-desmoside*. Differences in chemical properties between mono- and bis-desmosides may be exploited to facilitate their separation and isolation.

Four further sapogenols (C, D, F and G) were identified in soyabeans[35]. Soyasapogenols C, D and F have been shown to be artifacts of soyasapogenol B, which arise during acid hydrolysis. The complex interrelationships between these compounds have been rationalized by Price *et al.*[47].

Tsukamoto *et al.*[48] have found a mutant in *Glycine soja* which does not contain group A saponins. According to these authors[49], the variation in saponin composition in soyabean seeds is explained by different combinations of five genes controlling the utilization of soyasapogenol glycosides as substrates.

Soyasaponins are not only confined to soyabeans (*Glycine max*) but may also be found in other beans and peas. Price *et al.*[50] found soyasapogenol B in hydrolysates of the defatted kidney bean (*Phaseolus vulgaris*), haricot bean (*P. vulgaris*), runner bean (*P. aureus*), butter bean (*P. lunatus*), scarlet runner bean (*P. Coccineus)*, field bean (*Vicia faba*), lentil (*Lens culinaris*), chickpea (*Cicer arietinum*) and both green and yellow split pea (*Pisum sativum*). Analysis using thin-layer chromatography (TLC) and fast atom bombardment-mass spectrometry (FAB-MS) revealed soyasaponin I (Figure 1.5) to be present in all species. Ireland and Dziedzic[51] also detected soyasapogenol B in chickpea, haricot bean and red kidney bean. Price and coworkers[52] detected soyasaponin II (Figure

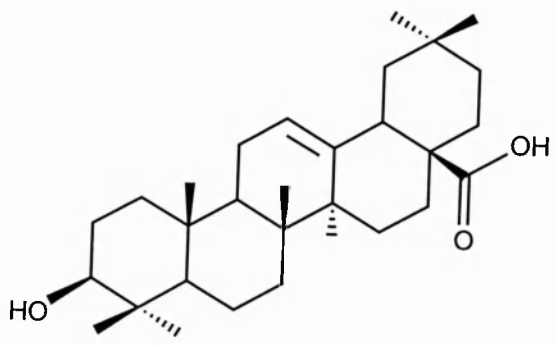


FIGURE 1.7. Structure of oleanolic acid.

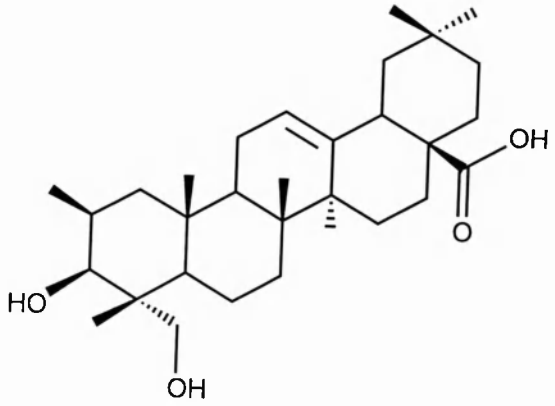


FIGURE 1.8. Structure of hederagenin.

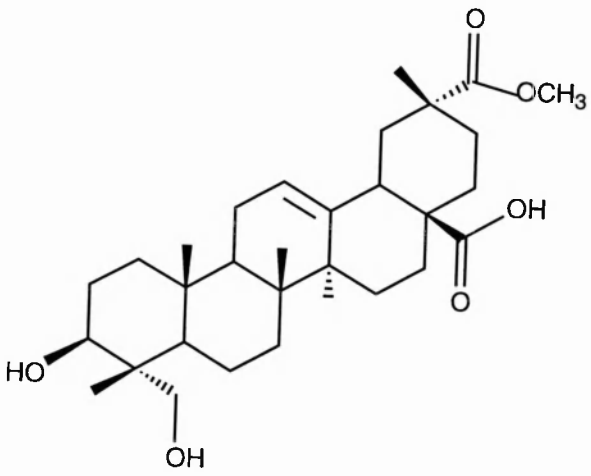


FIGURE 1.9. Structure of phytolaccagenic acid.

1.5) in runner bean and indicated the presence of a new saponin, containing soyasapogenol B as aglycone, in haricot, red kidney and scarlet runner beans. Curl *et al.*[53] have isolated this new saponin (soyasaponin V) (Figure 1.5) from haricot bean (*P. vulgaris*). Five other saponins, phaseollosides A to E, have also been isolated from *P. vulgaris*[54]. The saponins from *Vigna angularis*, azukisaponins, have been identified by Kitagawa *et al.*[55-57].

1.2.3.1.b Other plants

Saponins have been found in many hundreds of other plants used as human foods, and there is an abundance of information about their chemistry. The discussion here can not be comprehensive but it includes the chemical structures of the most common aglycones.

Quinoa, which is a food crop grown in the Andes, contain bitter saponins which have to be removed prior to human consumption. Mizui *et al.*[58] have characterized saponins present in the germ of quinoa, in which the aglycones are oleanolic acid (Figure 1.7), hederagenin (Figure 1.8) and phytolaccagenic acid (Figure 1.9).

Studies by Kawano *et al.*[59,60] have revealed steroidal saponins to be present in asparagus. Typical of the compounds isolated are asparasaponin I and II, which contain yamogenin (Figure 1.10).

Liquorice is one of the most widely investigated of the economically-important medicinal plants[61]. Large amounts of a mixture of saponins, primarily glycyrrhizin, which contains the aglycone glycyrrhetinic acid (Figure 1.11), are found in the roots of liquorice. This saponin is unusual because it has an intensely sweet taste, reportedly 50 times sweeter than sugar.

Many other saponins have been isolated and characterized from oats[62-64], onion[65], garlic[65], leek[65], peanut[52], tea[66-68], spinach[69], cucumber[70], pumkin[71], sugar beet[72], yam[73], fenugreek[74,75] and quillaja[76-78].

The presence of saponins has also been reported in a number of plants and seeds used locally for edible purposes or more widely as tonics and medicinally active herbs

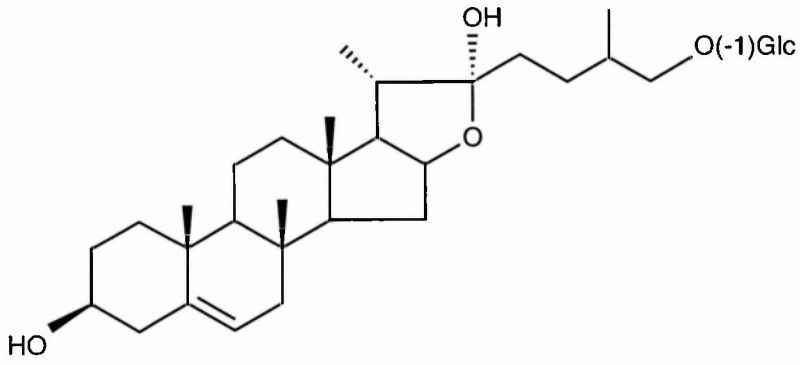


FIGURE 1.10. Structure of yamogenin.

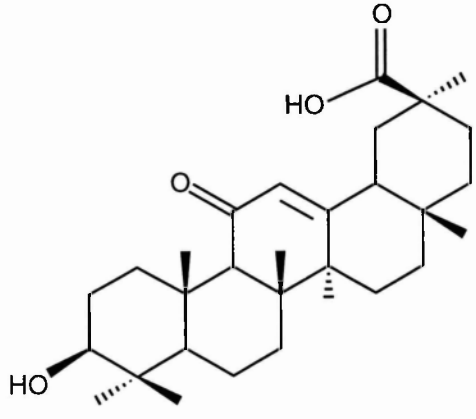


FIGURE 1.11. Structure of glycyrrhetinic acid.

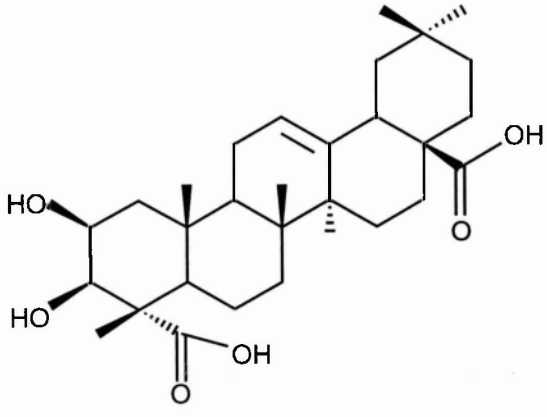


FIGURE 1.12. Structure of medicagenic acid.

such as ginseng, *Saponaria officinalis*, gypsophila, aloe, sage, sarsaparilla, guarana and nutmeg[35].

1.2.3.2 *Saponins in plants used for animal feed*

1.2.3.2.a Lupin

Three saponins, one containing soyasapogenol B and considered to be identical to soyasaponin I (Figure 1.5), the other two being bisdesmosides of soyasapogenol A, were detected in sweet and bitter *L. angustifolius*, *L. luteus*, *L. hispanicus*, *L. consentinii* and *L. mutabilis*[79]. No saponin was detected in *L. albus*. Cuadrado *et al.*[80] also found that *L. albus* had undetectable levels of saponins whereas soyasaponins I and II (Figure 1.5) were determined in both sweet *L. luteus* and bitter *L. mutabilis.*

1.2.3.2.b Alfalfa and other forage crops

Soyasaponins are found in the seed of alfalfa (*Medicago sativa*) but co-occur with additional saponins in the roots and above-ground parts. These additional saponins contain aglycones possessing carboxylic acid groups, notably hederagenin (Figure 1.8) and medicagenic acid[81,82] (Figure 1.12). Kitagawa *et al.*[83] have identified soyasaponin Be (Figure 1.6) in the aerial parts of American alfalfa together with glycosides of azukisapogenol. Oleszek *et al.*[84] have revealed glycosides of zahnic acid in Polish alfalfa.

Many of the clovers (*Melitotus alba*; *Trifolium pratense*; *Medicago hispida*) contain saponins, and there has been considerable interest in these because, as in alfalfa, it was once thought that they were responsible for ruminant bloat[36].

1.2.3.2.c Other plants

A number of saponins from sunflower (helianthosides)[85], horse chestnut (aescin)[86-89], and guar[90] has also been isolated and characterized.

1.2.4 Methods of extraction and structural elucidation

1.2.4.1 *Isolation and purification*

The isolation of pure saponins from crude plant extracts has in the past proved very difficult. Recently, a number of physico-chemical techniques have been developed which offer the possibility of providing such material, at least in favourable cases.

Saponins are generally isolated by extraction with organic solvents in a rather non-specific manner. Lipids and pigments may be removed by exhaustively extracting the dried or freeze-dried powdered plant tissue with acetone, hexane or chloroform. Subsequently, extraction of the residue with methanol removes saponins, together with low molecular mass species such as sugars, phenolic compounds, glycosides, oligosaccharides, and flavonoids. Separation of mono- and bis-desmosides has been achieved by extraction with methanol and water respectively, or by partitioning between butanol:water mixture[35-37]. Oleszek[91] has described a simple method for isolation and separation of saponins from alfalfa extracts; the saponins are selectively eluted from a C_{18} solid-phase column with aqueous methanol. Paper, electrophoretic, ion exchange, and adsorption column chromatographic methods have largely been overtaken by droplet counter current chromatography, high performance liquid chromatography, and so-called 'flash' chromatography[35].

Droplet counter current chromatography (DCCC)[92] has found application in the isolation and purification of saponins since no solid packing material is used and therefore no irreversible adsorption can occur. The technique, however, possesses limitations arising from the fact that the efficiency of the method depends entirely upon droplet formation; the resolution is not very high and long separation times are required[93]. Centrifugal thin-layer chromatography (CTLC) appears to be effective only for aglycones and saponins possessing one or two sugars[35]. 'Flash' chromatography[94], a simple and inexpensive hybrid of conventional column chromatography and high-performance liquid chromatography (HPLC), has been found to be an effective means of purifying and

separating saponins and other glycosides. The saponins and their acetyl derivatives from pea flour[95], guar meal[90], soyabeans[96] and haricot beans[53] have been isolated using reversed-phase flash chromatography.

However, in the majority of cases, no single technique is sufficient to yield pure saponins and combinations of the above, and other, techniques are necessary.

1.2.4.2 *Structure elucidation*

Until recently the structural elucidation of saponins has been conducted on the aglycone and (oligo)saccharide moieties resulting from acid hydrolysis. To minimize the possibility of artefact formation, developments in the use and in the interpretation of physical techniques such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) have enabled these to be applied to the analysis of hydrolysis products and intact glycosides.

1.2.4.2.a Hydrolysis

The chemical hydrolysis of saponins is usually carried out in 2M to 4M mineral acid. As an alternative, glucuronide linkages are also amenable to specific chemical cleavage using lead acetate and alkali[97], anodic oxidation and pyridine[98,99]. If an oleanane aglycone is attached directly to a uronic acid residue efficient cleavage has been achieved by UV irradiation in methanol[100]. Other workers have described enzymatic or microbial procedures[101].

The oligosaccharides are identified by conventional methods, the individual sugars being compared with standards and quantified by gas chromatography (GC) or HPLC[102]. Positions of linkage are usually identified by GC following permethylation and alditol acetate formation. The nature of the aglycone-sugar linkage is normally determined enzymatically, chemically or by inspection of ^{1}H or ^{13}C NMR spectra. A wide range of chromatographic and physicochemical methods has been employed for the structural elucidation of aglycones, these include infrared (IR) and ^{1}H or ^{13}C NMR

spectroscopy, mass spectrometry, optical rotary dispersion, circular dichroism, and *X*-ray crystallography[37]. The assignment of triterpene stereochemistry has been facilitated by lanthanide-induced shift and internuclear double resonance techniques[103].

1.2.4.2.b Physical methods

^{13}C NMR spectroscopy has been used for the examination of the glycosidic species and ^{1}H NMR spectroscopy applied to the determination (or confirmation) of the aglycone structure[35]. In recent years double resonance and other sophisticated techniques have been employed for the analysis of saponin structure either directly, without a requirement for prior hydrolysis or derivatization[104], or following peracetylation which has been shown to reduce problems of peak broadening in the ^{1}H spectra due to exchange of hydrogen between sample and solvent[105].

Not only can the individual sugars linked to the aglycone be distinguished by ^{13}C NMR spectroscopy, but the number of anomeric carbons is readily determined and defines the number of individual sugar molecules present. The technique also allows sugars of different ring sizes to be identified[106] and the site of acetylation of a sugar moiety to be ascertained[107]. The anomeric configuration of individual sugars in a saponin can be determined by ^{13}C NMR spectroscopy. Both the chemical shifts of α- and ß-anomeric carbons and the direct bonded C to H coupling constants of the C_1 signal are useful in this respect since the substitution of an aglycone-OH group by an *O*-sugar moiety results in shifts in the α-carbon and those adjacent to it. Thus, a comparison of the ^{13}C NMR spectra of a saponin, prosapogenin, or sapogenin will reveal the point of attachment of the sugar to the aglycone[35].

^{1}H NMR spectroscopy has an important role in the analysis of the aglycone structure where a variety of techniques may be applied, including shift reagents, decoupling, etc. to facilitate the analysis[35].

Mass spectra depict the relationship between chemical bond lability and fragment

stability through the mass and abundance of the component ions. Modern mass spectrometry can provide both the relative molecular mass and primary structure on sub-milligram amounts of compounds such as saponins. Conventional electron ionization (EI) as well as chemical ionization (CI) mass spectrometry was of limited use for saponin analysis because of its need for sample volatilization[37]. In recent years, however, the area has been increasingly dominated by newer ionization techniques such as field desorption (FD), plasma desorption and FAB.

Examination of saponins by FD-MS not only provides direct information about the molecular mass but also facilitates the elucidation of polysaccharide structure since the cleavages of the individual sugars have been successfully interpreted[108]. FAB, in which the sample, usually in a glycerol matrix, is bombarded at ambient temperatures by a beam of neutral xenon atoms has been applied to the analysis of plant saponins[109,52]. The complementary nature of the information which can be obtained from inspection of the positive and negative ion FAB-MS has been discussed by Fraisse *et al.*[110]. Price *et al.*[52] illustrated the value of the method by analyzing crude methanolic extracts of defatted legume flours. Not only were the expected soyasaponins observed in these flours, but the presence of additional saponins was indicated and this analysis greatly facilitated subsequent isolation and characterization[53]. FAB-MS may also be applied in the quantitative mode[111] and this technique has been used for the determination of the glycoalkaloid α-tomatine in tomato fruit[112].

Positive-ion liquid secondary ion mass spectrometry (LSIMS), and linked scanning at constant B/E (MS/MS) have been recently used to determine the chemical structure of soyasaponin I isolated from mungbean[113].

The use of combined high-performance liquid chromatography-mass spectrometry (HPLC-MS) for the direct separation, analysis (both qualitative and quantitative) and structural elucidation of saponins is awaited with interest. Hattori *et al.*[114] have utilized HPLC-MS for the screening of saponins in crude oriental drugs. The method shows clear potential although specific clean-up procedures may be necessary for individual samples.

1.2.5 Quantitative methods

Methods for the qualitative and quantitative estimation of saponins in plant tissue are very varied. They include the measurement of simple physical properties of saponins, such as their foam-forming ability, and of biological properties, such as their haemolytic and antifungal effects, to the use of modern techniques employing chromatographic and spectrophotometric methods. In addition, there are various bioassays which have been optimized for the analysis of particular groups of saponins.

1.2.5.1 *Early methods*

Reliable quantitative estimation of the saponin content of plant material has proved very difficult in the past, and any data obtained before 1980 are at best only approximate.

A useful indication of the saponin content of plant material, although only a lower limit, can be obtained by determining the yield of purified saponin gravimetrically[115,116]. Other simple methods exploit the characteristic physical properties of saponins, such as their foaming power[117] or their haemolytic activity[118]. Spectrophotometric methods have been reported which exploit the colours produced by the reactions of saponins with vanillin[119] or anisaldehyde[120]. Although very sensitive, these methods are not suitable for estimating saponins in crude plant extracts because the reactions are not specific, and coloured compounds can be produced from other compounds such as phytosterols and flavonoids[36]. The vanillin method has, however, been used to estimate the quantity of ginseng saponins in candies and jellies[121]. Bioassay methods have also been used extensively and include toxicity to fish[122], inhibition of germination of seeds[123], and inhibition of growth of the mould *Trichoderma viride*[124]. This last method has been developed specifically for alfalfa saponins.

1.2.5.2 *Thin layer chromatography (TLC)*

Quantitative TLC is a well-established technique which has been applied to saponin estimation by several workers[125-127,50]. The essence of the technique is to spot a TLC plate with a crude saponin extract, develop the plate with a suitable solvent system, and use one of a number of methods for estimating the quantity of saponin on the plate. Wolf and Thomas[128] evaluated 22 different solvents systems for the separation of soyabean saponin on silica gel. Of these, the two that appear to have been most used are *n*-butanol: ethanol: concentrated ammonia (7:2:5) and chloroform: methanol: water (65:35:10). The former is the basis for the quantitative estimations reported by Andrzejewska[129] and Fenwick and Oakenfull[125]. However, the results quoted in the latter paper have been questioned by others[130,127]. Kitagawa *et al.*[131] have used the chloroform: methanol: water solvent system in conjunction with silica gel impregnated with oxalic acid to produce an impressive separation of soyasaponins I, II, III, A_1 and A_2. Curl *et al.*[127] added a reversed-phase clean-up stage and a modified spectrophotometric estimation procedure to the method of Fenwick and Oakenfull[125]. Saponin levels in soya measured in this manner were in close agreement with those obtained by GC and HPLC[127,131]. More recently, Ng *et al.*[132] have also successfully developed a TLC method for the analysis of total saponin content and composition of quinoa plant tissue using reversed-phase silica plates and methanol/water as elution system.

Visualization sprays for the development of thin-layer chromatograms of extracts of plant saponins include Carr-Price reagent, Liebermann-Burchard reagent, phospho-tungstic acid, 1% $CeSO_4$ in 10% H_2SO_4, 10% H_2SO_4 in EtOH, 50% H_2SO_4, phenol, H_2SO_4, 0.5% *p*-anisaldehyde, 1.0% H_2SO_4 in AcOH[35].

Modern developments in the production of reversed-phase and other modified silica gel layers has generated the possibility of increased resolution for the separation of intact saponins, but, more importantly, has enabled the complete separation of saponins from the associated oligosaccharide material. Amarowicz and co-workers[133] have found

that separation of saponins with similar polarities is much more improved when using high-performance TLC (HPTLC) plates than in case of using TLC plates.

1.2.5.3 *Gas chromatography (GC)*

Gas chromatography has been used almost exclusively for the separation and analysis of the aglycone moiety of the saponin molecule as its trimethylsilyl ether. The levels of total saponin in various varieties of soyabean were investigated by Kitagawa *et al.*[130] who measured soyasapogenols A and B after silylation with *N,O-bis*(trimethylsilyl)trifluoro-acetamide in pyridine. Since in this case the ratio of sapogenol/saponin is known, one can readily convert the results of such analysis to saponin content. Bombardelli *et al.*[134] reported a method whereby the intact ginseng saponins were silylated and analyzed by GC. The analysis of trimethylsilylated triterpenoid glycosides containing four or less sugar units was considered satisfactory using this system. Martinelli[135] has reviewed the application of coupled gas chromatography-mass spectrometry (GC-MS) in the electron ionization mode for the investigation of plant glycosides (including saponins) as their trimethylsilylethers, and showed that saponins containing sugar moieties linked to the aglycone through an ester linkage underwent a deglucosidation reaction in the injection port of the GC.

The use of FAB-MS and GC has allowed the levels of saponins containing soyasapogenols A and/or B to be ascertained in soya bean, *Phaseolus* species, lentils, chickpeas, peas[50] and lupin[79,80]. Likewise, the use of both techniques has also allowed the levels of saponins containing oleanolic acid, hederagenin, and phytolaccagenic acid to be determined in quinoa[136,137].

1.2.5.4 *High-performance liquid chromatography (HPLC)*

High-performance liquid chromatography, because it can deal effectively with involatile, polar compounds, has been used extensively for the separation and quantification of both intact saponins and their sapogenins. The main problem with the use of this technique is

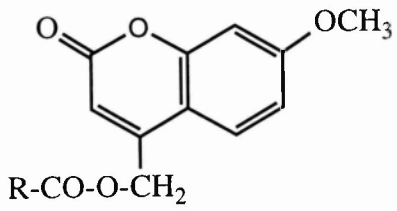


FIGURE 1.13. Structure of the coumarin derivatives of soyasaponins[130].

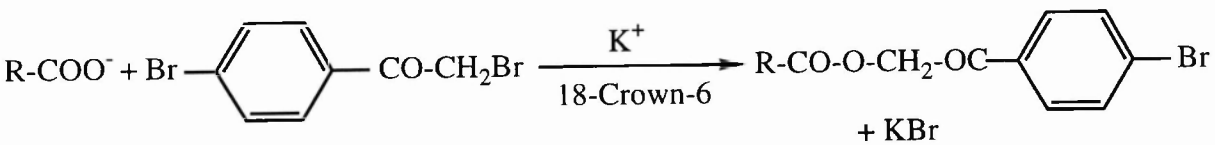


FIGURE 1.14. Derivatization of saponins according to Oleszek *et al.*[141].

the lack of sensitivity of most of the intact saponins and their sapogenols to conventional ultraviolet (UV) detectors.

Methods for the analysis of sapogenins have employed both normal and reversed-phase chromatography on derivatized and underivatized mixtures. Soyabean sapogenins have been determined using HPLC by Ireland and Dziedzic[138], the authors then assuming a sapogenin/carbohydrate ratio of 1:1 in order to calculate saponin content. The same group has separated intact soyasaponins by HPLC with mass (evaporative light scattering) detection but quantification was not achieved[139]. Damon *et al.*[140] have employed small gradient changes in the eluting solvent (methanol:water, acetonitrile: water) on reversed-phase columns to separate oleanane saponins, while Kitagawa *et al.*[130] have converted soyasaponins to their coumarin derivatives (Figure 1.13) by esterification of the carboxylic acid moiety, prior to separation and quantification on reversed phase C_{18} columns, with fluorescence detection. Shiraiwa *et al.*[44,45] have determined the composition and structure of groups A and B saponins in soyabean seed by HPLC using a reversed-phase silica column with a acetonitrile-propanol-water-acetic acid mixture. Detection was monitored by UV absorption at 205 nm.

Oleszek *et al.*[141] have separated the hederagenin and medicagenic acid-containing saponins from alfalfa leaf by HPLC, after initial reaction with 4-bromophenacyl bromide (Figure 1.14). Analysis of saponins in oat kernels has also been accomplished by HPLC using an octyl-silica column and gradient elution with acetonitrile in water[142]. Glycyrrhizin possesses a conjugated dienone moiety and this chromophore has been used as the basis of quality control and screening methods based upon HPLC[143].

The extraction and isolation of twelve ginseng saponins have been carried out by Choi and co-workers[144] using a combination of preparative HPLC on silica gel carbohydrate and reversed-phase columns, and refractive index detection. Soldati[145] and Kanazawa *et al.*[146] have separated ginsenosides and monitored the HPLC eluate at 203 nm. More recently, Park *et al.*[147] have reported the microanalysis of ginseng saponins by

ion chromatography with pulsed amperometric detection (PAD). During the amperometric detection process, oxidation products of analyte accumulate on the surface of the electrode, resulting in a decrease in sensitivity and reproducibility with time. PAD was developed to overcome this drawback by applying both oxidation and reduction potentials after the measuring potential as a cleaning process for the electrode. Detection sensitivity and reproducibility were found to be greatly enhanced by this pulse sequence.

1.2.5.5 *Other methods*

Micellar electrokinetic capillary chromatography has been investigated for characterization of hapten-protein conjugates. Frøkiær *et al.*[148] isolated soyasaponin I from pea and used it as hapten for production of anti-saponin specific polyclonal antibodies. Soyasaponin I was coupled to Kunitz soyabean trypsin inhibitor and bovine serum albumin. The degree of coupling was determined by high-performance capillary electrophoresis (HPCE). The same authors[149] have developed an enzyme linked immunosorbent assay (ELISA) for quantification of soyasaponin I in pea flour.

1.2.6 Saponin content

If a reliable estimate of saponin content is to be made, an appropriate analytical method will, to a considerable degree, depend upon the chemical and biological properties of the saponin(s) present. The saponin contents of soyabeans, peas and beans are summarized in Table 1.2. The figures quoted are intended to be indicative of the general levels.

TABLE 1.2. Saponin content (%)(dry weight) of soya, peas and beans.

Soya bean	0.22-0.33[a], 0.22-0.35[b], 0.47[c], 0.65[d]
Dried pea	0.18[d]
Mung bean	0.05[d]
Runner bean	0.34[d]
Butter bean	0.10[d]
Kidney bean	0.35[d], 0.2[e]
Haricot bean	0.41[d]
Field bean	0.01[d]
Broad bean	0.35[d]
Lentil	0.11[d]
Yellow split pea	0.11[d]
Chickpea	0.23[d]

[a]HPLC method: Kitagawa *et al.*[131]
[b]GC method: Kitagawa *et al.*[130]
[c]HPLC method: Ireland *et al.*[138]
[d]GC method: Price *et al.*[50]
[e]HPLC method: Ireland and Dziedzic[51]

On the basis of an analysis of various edible beans, Price *et al.*[50] concluded that soyabean contained the highest levels of saponins, although detailed examination of the effects of genetic and agronomic variation had, at that time, not been conducted. Haricot, runner, and kidney beans appeared, after soya, to contain most saponins, although lentils, chickpea, broad beans, and green pea were all reported by others to possess very high levels[35].

Alfalfa has levels that may be < 0.3% (dry weight), in the case of "low" or sweet alfalfa, or much higher (typically > 1.5%) in the case of high saponin cultivars[35].

'Bitter', 'semi-sweet', and 'sweet' varieties of quinoa occur and exhibit a significant gradation in total saponin content[37]. The same finding is true for different species of 'bitter' and 'sweet' lupin[79].

1.2.6.1 *Factors affecting saponin content*

In addition to the plant examined, its physiological age, and state are important to the saponin content, as are the environmental and agronomic factors associated with growth of the plant, and post harvest treatments, including storage and processing. Thus,

Kitagawa and co-workers[130,131] found a variety of soyabean from China to be richer in soyasaponins than those from Japan, Canada, or the U.S. Evidence of variation in saponin content has also been found within lentils, moth bean, alfalfa and quinoa[37]. Cuadrado *et al.*[80] have analyzed the saponin content and composition of Andean crops and concluded that environmental conditions in the Peruvian highlands are determinants of both the amount and composition of saponins present in bitter lupin and quinoa.

Fermentation reduced the saponin content of soya[130,131] and alfalfa[150]. Studies on the effects of cooking or processing of beans and peas on saponin content are very few and contradictory. For instance, cooking and canning have little effect on levels in broad beans and navy (baked) beans[126], but cooking appears to have a significant effect in reducing the saponin content of moth beans[151], chickpeas and black grams[152].

The levels of saponins in quinoa, which are mainly concentrated in the outer layers of the grain, have been significantly reduced using abrasive dehulling[137].

Sprouting, as a means of reducing saponin content, produces variable results; for instance, saponins are retained in both sprouted mungbean and alfalfa[126], but are reduced when moth bean[151], chickpea[152], blackgram[153], rice bean[154], faba bean[155] and pea[156] are sprouted. However, the non-specific nature of the analytical methods employed suggest that these investigations would benefit from repetition[37]. Light was found[157] to have a significant effect on the saponin content of germinated soyabean; saponin levels increased under light irradiated germination, but decreased when light was excluded.

Tani *et al.*[158] have examined the distribution of total and individual saponins in soyabean seed. The majority of the soyasaponins was concentrated in the parts of the seed (plumule, hypocotyl, radicle) that develop into the mature plant. Shimoyamada *et al.*[159] found aerial parts of the plant to be richer in saponins than seed hypocotyl > stem, branch, and petiole. In the underground parts, soyasaponin I level decreased in the order root hairs > lateral > main root. The same authors[160] observed that, in developing soyabean seed, the saponin accumulation was completed before the endpoint of fresh and

dry weight increases. Both the saponin content and composition of soyabean seeds have been found to be more dependent on the variety than on the cultivation year[161].

Önning *et al.*[162] have found the oat kernel saponins to be situated mainly in the endosperm. More recently, these authors have studied the degradation of oat saponins during heat processing together with the effect of pH, stainless steel, and iron at different temperatures. Heating at 140°C, especially at pH 4, led to partial destruction of the oat saponins. Addition of catalytic amounts of iron and stainless steel dramatically increased the rate of saponin breakdown[163].

The amount and type of alfalfa saponins are greatly affected by both the age and the part of the plant examined. The average saponin content of leaves and seeds in alfalfa have been found to be twice that in stems and flowers, and a significant reduction occurred in the saponin content of older plants[92,164]. Levels of saponin in root material have been found to be greater than that in the top leaves. Livingstone *et al.*[165] have reported the saponin content of varieties of alfalfa grown commercially in the U.S. Levels ranged from 0.1 to 1.7% (dry weight). The same authors found the saponins of alfalfa meal to be concentrated in the protein fraction.

The amount of glycyrrhizin in roots and underground stems of liquorice is markedly affected by variety, source, and climatic conditions[61]. Függersberger-Heinz and Franz[166] have shown the concentrations to be higher in the main roots than in the lateral stems. These authors also found no detectable glycyrrhizin in the green parts of the plant. Hayashi *et al.*[167] detected soyasaponins in different organs of liquorice, particularly in the seed, hypocotyl and rootlet, whereas glycyrrhizin was detected exclusively in the thickening root and the stolon.

The root age and its subsequent processing are crucial for the production of high-quality ginseng. Koizumi *et al.*[168] examined the distribution of ginsenosides in *Panax ginseng* and found levels were lowest in the leafstalks and stem, intermediate in the main root and lateral roots and highest in the leaves and root hairs.

The saponin content of root, seed and pod-shell of guar was higher than the

leaves and stem[169]. These authors[170] have also found that the saponin content of fenugreek seeds increases towards maturity and decreases at maturity of both seeds and pod walls.

1.2.7 Physical properties

The potential for the use of certain saponins as 'natural additives' is considerable given their physical and physicochemical properties and the prevailing 'green' attitudes amongst consumers which favour the use of such products rather than 'synthetic additives' in food.

1.2.7.1 *Solubility*

Saponins generally are readily soluble in water, methanol, and aqueous ethanol. They are, however, virtually insoluble in such solvents as acetone, or diethyl ether[36]. Shimoyamada *et al.*[171] has measured the solubility of soyasaponin I in aqueous ethanol and buffer. Soyasaponin I showed the highest solubility in 60% ethanol, and was more soluble in the alkaline region in buffers prepared at various pHs. The addition of acetyl-soyasaponin A_1 increased the solubility of soyasaponin I in buffer.

1.2.7.2 *Aggregation*

Because saponins are amphiphilic, they form micelle-like aggregates in water. Like many other amphiphiles, they appear to have critical micelle concentrations (CMC). Below this concentration the molecules remain unassociated, and the transition to the micellar state is marked by an abrupt change in some physical property of the solution such as surface tension. The self-aggregation of saponins has been described[36]; the extent of aggregation depends upon the saponin structure. Whereas soyasaponins yield only dimeric structures, those from *Gypsophila* and *Saponaria* form larger aggregates, perhaps ten and fifty

molecules, respectively.

1.2.7.3 *Formation of mixed micelles with bile acids*

The formation of mixed micelles between saponins and bile acids is a property having far reaching consequences; this mechanism having been proposed for the anti-fungal properties of many saponins, and for many of the effects observed in insects, animals, and man. Whereas the size of self-aggregating saponin structures is limited by the size of the bulky oligosaccharide moieties, and those of bile salts alone are limited by electrostatic repulsions of the acid groups, in the mixed micelles these destabilizing factors are relieved and greatly extended stacks, with saponin and bile acid molecules alternating, are produced[36]. Oakenfull[172] has examined the sizes and structures of these micelles in detail. Current opinion suggests that the micelles contain a loose internal structure with the sugar moieties being both on the surface and within the interior. There is currently considerable interest in such structures as adjuvants in the veterinary and pharmacological areas[37].

1.2.7.4 *Saponins as emulsifiers*

Saponins are strongly surface active, and might, therefore, reasonably be expected to be good emulsifiers. Gohtani *et al.*[173] have reported in detail on the foaming power, emulsification properties, and surface activity of commercial soyasaponin and soyasaponins Ab, deacetyl Ab and Bb. The bark of the soaptree, *Quillaja saponaria*, contains approximately 10% saponin and has been used as a foaming agent in confectionery, baked goods, and beverages[37].

1.2.8 Metabolism and human exposure

There is a lack of detailed information on the fate of saponins within the gut of monogastric animals. Gestetner *et al.*[174] fed soyabean meal to chicks, rats, and mice and observed that whilst saponins were present in the small intestine only sapogenols could be found in the caecum and large intestine. Odani *et al.*[175] have studied the fate of ginsenosides Rg_1 and Rb_1 in the rat. When administered orally Rg_1 was broken down in the stomach and small intestine to six products, identical to the behaviour of the compound with weak acid. Only a trace (1-2%) of glycyrrhizin administered orally or by intravenous injection was excreted intact or as the aglycone[176]. The appearance of the latter was rather more rapid following oral administration; this finding is consistent with the hydrolysis of the saponin via gastric juice, intestinal bacteria, and intestinal enzymes. The aglycone is able to react readily with serum albumin and is hence rapidly excreted. Following intravenous administration of glycyrrhizin to the rat most of the saponin was found in the plasma and blood after 6 minutes; no glycyrrhizin could be detected in the brain whilst the lung, heart, stomach, and small intestine all contained intermediate amounts[177]. The highest concentration of glycyrrhetinic acid was found in the plasma and blood, smaller amounts being present in the brain and other organs.

Information on saponin intakes is very limited. Ridout *et al.*[178] have calculated mean daily intakes on the basis of limited screening of food products and information on the UK total diet. These intakes (see Table 1.3) are thus only approximate; however, they do serve to illustrate the large variation due to the composition of the diet and to the levels of saponin-containing foods actually consumed. This dietary difference is also responsible for significant differences in the consumption of saponins with various structures (and therefore varying biological activities).

TABLE 1.3. Calculated UK mean daily intakes (mg person^{-1}) of saponins.

Subgroup	
Family	15
Children	13
Vegetarian	110
Males only:	
Caucasian	10
W. Indian	47
Asian	170
Asian (vegetarian)	214

1.2.9 Physiological and pharmacological properties

The toxic nature of many saponins has been known and exploited for many centuries, particularly their toxicity to cold-blooded animals such as molluscs, amphibians, and fish. Only recently, however, have the full range and complexity of the effects that these compounds can exert upon biological systems begun to receive systematic investigation.

1.2.9.1 *Interaction with biological membranes*

Saponins have long been known to cause lysis of erythrocytes *in vitro*. Indeed the quantifiable release of haemoglobin, coupled with the inhibition of this effect by cholesterol, has been extensively used as a means of detecting and 'quantifying' saponins in plant material. The primary action of membranolytic saponins upon cells is to increase the permeability of the plasma membrane. This leads to loss of essential electrochemical concentration gradients and to the inevitable destruction of the cell. There is considerable evidence to support the irreversible binding of membranolytic saponins to discrete sites within the plasma membranes; however precise details of the interactions involved are still sought[37]. There is very clear variation both in the haemolytic activity of individual saponins, and in the order of susceptibility within the animal kingdom. Generally the guinea pig and horse are most susceptible[36].

It has been suggested that saponins combine permanently with membrane cholesterol to form permeable, micelle-like aggregates within the plane of the

membrane[179]. The precise molecular structure of these lesions remains unclear, although in one model[180] the saponin molecules are arranged in a ring with their hydrophobic moieties combined with cholesterol around the outer perimeter. It has also been suggested[181] that, rather than being a specific site for saponin binding, cholesterol exerts its effect by altering the structure of the membrane.

Haemolytic saponins significantly increase the permeability of isolated intestine *in vitro*[182-184]. Although it is considered that the nature of the aglycone is crucial, with increasing numbers of polar (carboxylic acid) groupings enhancing permeabilizing activity, the oligosaccharide moiety is also important. Branching of the sugars may be a significant structural feature[37,185].

The study of the mechanisms involved in this complex area would seem currently to be facilitated by the availability of well-defined and purified saponins and by the various techniques which may be employed for their chemical or enzymic modification. Takechi and Tanaka[186] have examined, for example, the structure-related properties of α-hederin (obtained from *Hedera rhombea*) and its derivatives; the data obtained indicated that the terminal rhamnose unit of the disaccharide moiety was more important for antifungal activity than for haemolytic activity, whilst the latter was more affected by the presence of a free carboxylic acid group in the hederagenin aglycone. More recently, these authors[187] have studied the haemolytic and antifungal activities of synthetic saponins. Both activities of the steroid glycosides were generally parallel to each other, while almost all haemolytic triterpenoid glycosides showed no antifungal activity.

1.2.9.2 *Effects on enzymes and metabolism*

Despite the fact that saponins have such powerful metabolic effects in many organisms, few studies have been made of their direct effects on enzyme activities. Alfalfa saponins have been shown to inhibit respiration in the isolated rat diaphragm which can be explained by inhibition of succinate oxidase in the liver[36]. Ginseng saponins have been reported to inhibit Na^+, K^+-ATPase from dog cardiac sarcolema[188]. The saponins from

licorice potentiate the action of the glucocorticoids, and this appears to be related to inactivation of the hormones by certain liver enzymes[189].

Turning to more general metabolic effects, ginseng saponins have been found to have powerful effects on carbohydrate and lipid metabolism[190], and increase protein synthesis in the liver, along with increased synthesis of RNA and increased RNA-polymerase activity[191]. More recently, Potter *et al.*[192] have studied the chemical interactions between proteins and saponins and their results indicated that the effect of saponins on serum lipid profiles is dependent on the source of dietary protein.

1.2.9.3 *Sensory properties of saponins*

It is generally believed that saponins lend a bitter flavour to foods. Soyasaponin I, isolated from pea, has been shown to possess a characteristic flavour which is perceived as 'bitter' or 'astringent' by human subjects[193], and there is a linear relationship between the strength of the sensory response and the concentration of the saponin in water over the range 0 to 0.1% (weight/volume). Soyasaponins also adversely affect the palatability of soyabean products. Several studies carried out by Kudou *et al.*[194] suggest that the improvement of the flavours of soyabean foods is possible via enzymic hydrolysis of soyasaponins. These authors[194] found that soyabean saponin Bb content was less and sensory properties were better in miso made from dehulled soyabeans than in that made from whole soyabeans. However, Okubo *et al.*[195] have reported group A saponins to be the main factors responsible for bitterness and astringency in soyabeans.

The processing of quinoa also leads to changes in sensory characteristics; removal of saponins by washing and abrasion was associated with reductions in bitterness and astringency[137]. On the other hand, the principle saponin of liquorice root, glycyrrhinic acid, is reported to be 50 times sweeter than sucrose. It therefore provides an important contribution to the characteristic flavour of liquorice and is commonly used as a sweetening additive[196].

1.2.9.4 *Effects on viruses*

Saponins from horse chestnut and *Thea sinensis* have been found to possess activity against influenza A2 virus[197], whilst glycyrrhizin is active against several DNA and RNA viruses and varicellazoster virus[198]. An interesting observation regarding the behaviours of group A and group B saponins from soyabeans has been made by Nakashima *et al.*[199]. Whilst both the group A and deacetylated group A soyasaponins showed inhibitory effects towards HIV infection *in vitro*, these were small as compared with the behaviour of the group B saponins. Since the latter are found widely distributed throughout the soyabean plant, as well as amongst members of the Leguminosae generally, it is tempting to speculate that the group B saponins have important physiological roles to play in leguminous plants. On the other hand, group E saponins have been reported to inhibit the activation of Epstein-Barr virus early antigen[200].

Saponins have been utilized as adjuvants in an anti-malarial and rabies vaccines[201]. Morein and co-workers[202] have described the formation of ISCOMs (Immuno Stimulating COMplexes) and have applied these in commercial vaccines. Bomford *et al.*[203] have examined the adjuvant activity and ISCOMs formation by a series of saponins. The only two saponins apart from *Quillaja* that were adjuvant-active were *Gypsophila* and *Saponaria*. While *Saponaria* saponins formed irregular ISCOM-like structures, *Gypsophila* produced a sheet of joined pore-like structures.

1.2.9.5 *Effects on fungi, insects and fish*

It has been suggested that saponins are associated with plant protection and disease resistance because of their distribution in tissues most vulnerable to mould attack or insect predation. However, the exact biological and physiological role of these secondary metabolites in plants remains obscure.

Amongst the findings reported by Appelbaum and Birk[204] and Birk and Peri[205] were several which suggested that alfalfa saponins were more active than soyabean saponins against fungi and insects. The toxicity of oat roots, root-extracts, and isolated

saponins to *Pythium* and other fungi has also been reported[206]. According to Levy *et al.*[207], the inhibitory activity of alfalfa saponins towards *Sclerotium rolfsii*, a plant pathogenic fungus, was dependent upon the presence of a potentially free C-3 hydroxyl group in the saponin aglycone. The antifungal activity of medicagenic acid and its derivatives against *Trichoderma viride* was found to be markedly dependent upon free CO_2H and OH groups[82,208].

Oakenfull and Sidhu[36] have summarized the effects of saponins on insects, molluscs, and aquatic vertebrates. Thus, for example, alfalfa saponins are active against the larvae of the grass grub[209,210] whilst soyasaponin is reported to be highly toxic to the rice weevil.

The influence of six levels of bruchids infestation on the saponin content of three pulses (chickpea, red gram, and green gram) has been studied by Modgil and Mehta[211]. Saponins were found to increase with the increase in the level of infestation.

When saponins are added to water, fish may be rapidly paralysed and die. This toxic effect of saponins to fish is a property which has been exploited by man since ancient times, and in more recent times has been the basis for saponin bioassays based upon the response of snails, tadpoles, and other small fish. Dorsaz *et al.*[212] have searched for natural molluscides which may be of value in controlling water snails, which are vectors of schistosomiasis, an endemic disease in parts of Africa. Saponins in a number of African plant species have been shown to be active in this respect. Khalil and El-Adawy[213] have recently developed a fish mortality assay to measure toxicity of crude saponins from peas, beans, and soyabeans. As well as being potent haemolysins, saponin extracts were lethal to guppy fish.

1.2.9.6 *Effects on birds and mammals*

Despite the high toxicity of many saponins when given intravenously to higher animals[35,36], their toxic effects are greatly reduced when administered orally. The generally low oral toxicity of most common saponins has led to the frequently stated

assumption that they are absorbed to a negligible extent by mammals. Nevertheless, haemolytic saponins readily interact with and permeabilize the small intestinal mucosal cells of mammals *in vitro* (see Section 1.2.9.1) and it would be surprising if a similar effect did not occur in the intact animal. One consequence of this may be that the permeabilized cells will be readily eliminated by the normal processes of epithelial replacement. This would explain the failure of haemolytic saponins to cross the gut mucosa, but the physiological consequences of increased permeability and mucosal turnover require further investigation. However, it can perhaps be assumed that the low toxicity of orally ingested saponins to man and other large mammals is due primarily to the large surface area of the gastrointestinal tract in relation to the concentration of saponins to which it is exposed[35,37].

Ishaaya *et al.*[214] could detect no adverse effect of high concentrations of soyabean saponins fed to chicks, mice, and rats, although it has subsequently been claimed that the goitrogenic activity of soyabean products observed in rats is at least partly due to soyasaponins[215]. Intrarumial administration of alfalfa saponins to sheep resulted in a reduction in nutrient degradation and microbial fermentation in the rumen[216]. Prolonged administration of disodium glycyrrhizinate to mice produced no evidence of toxicity[217]. However, glycyrrhizin is known to mimic the action of the mineralocorticoid hormone, aldosterone, and hence to cause retention of water and sodium[218]. A *Quillaja* saponin mixture incorporated in feed at 1.5% by weight did not produce any toxic effect in mice for either short term or prolonged periods[219].

The growth-retarding effect of a variety of saponin-containing feedingstuffs has been commented on by numerous workers[35,36]. Given the bitterness of the vast majority of saponins it is tempting to suggest that the primary effect of saponins is to reduce the palatability of diets. In support of this, it has been noted that many species are sensitive to flavours perceived by humans as bitter, and that foods rich in saponins are avoided by such species[220,221]. Conversely, the addition of cholesterol to the diet at levels which abolish the growth inhibiting effects of saponins does not reduce bitterness or palatability.

Saponins may, however, inhibit growth by reducing the availability of essential nutrients. For example, there was evidence of binding between ammoniated glycyrrhizin, alfalfa saponins, and zinc[222]. Southon *et al.*[223] have shown soyabean saponins to produce similar effects; reduced zinc availability is associated with anorexia and growth inhibition in experimental animals. In contrast, it has recently been reported that ginseng saponins increased the absorption of iron when added to basal feed of rats at levels between 0.02 and 1.5% by weight[224].

Differences in the effects of saponins observed in ruminants and non-ruminant animals have been attributed to alterations in metabolism brought about by ruminal micro-organisms[35,36].

1.2.9.7 *Hypocholesterolemic effects*

There is abundant evidence that saponins do interact with sterols in the gastrointestinal tract in a way which might prove beneficial to humans. The hypocholesterolemic effect of some saponins has therefore provoked considerable clinical interest. To investigate the mechanism underlying the hypocholesterolemic effect of alfalfa, Malinow *et al.*[225] investigated the effects of isolated alfalfa-top saponins on cholesterol absorption in rats. A progressive inhibition of cholesterol absorption with increasing saponin dosage was observed. The same authors[226] have shown that a reduction in experimentally-induced hypocholesterolemia, brought about with saponins, leads to changes in systemic cholesterol metabolism which can beneficially alter the progress of cardiovascular disease. The possibility that such an effect may be of benefit to man is strengthened by some other work with primates[227]. Alfalfa saponins appear not to be toxic at the levels necessary to produce a useful physiological response. Improved plasma cholesterol levels have been measured in a group of hyperlipidemic human subjects fed alfalfa seeds[228].

Apart from binding, and hence limiting the absorption of dietary cholesterol, certain substances have the capacity to alter cholesterol metabolism by interfering with the enterohepatic bile salt circulation. Oakenfull and Topping[229] reported that saponins

facilitate the adsorption of bile salts to non-digestible macromolecular substrates *in vitro*. However, Calcert and Yates[230] were unable to show any effect of saponins upon the binding of bile salts by various constituents of dietary fibre. Rotenberg and Eggum[231] also concluded that saponins play no part in the hypocholesterolemic activity of pectin. Other studies (see Section 1.2.8.3) demonstrated that saponins form large micellar aggregates with bile acids. The formation of these structures significantly reduces the absorption of bile salts from the perfused small intestine of the rat[232].

The importance of saponins in the hypocholesterolemic effect of alfalfa is thrown into question by the finding of Story *et al.*[233] that the binding of bile salts by alfalfa plant material *in vitro* is not reduced by the extraction of saponins, and that the ability of alfalfa to reduce the accumulation of liver cholesterol in cholesterol-supplemented rats was significantly increased by the removal of saponins.

There is further controversy in relation to the significance of the hypocholesterolemic effect of soyabean saponins. A study with a group of normal human volunteers showed that the saponins present in soyabean flour significantly increased faecal excretion of bile acids[234]. The hypocholesterolemic effect of soyabean protein fed to rabbits was not influenced by supplementation with an undefined commercial saponin, and this led the authors to suggest that the composition of the protein itself may be of primary importance in the modification of cholesterol metabolism[235].

Much of the previous uncertainty as to the usefulness and importance of saponins in the control of cholesterol metabolism in man appears to be due to the fact that saponins have often tended to be treated as a single, well-defined substance of known concentration. However, the term 'saponin' is often only an operational definition applied to mixtures of substances whose purity and chemical structure are unknown[236].

1.2.9.8 *Other effects*

Glycyrrhizin and its aglycone[237], oleanolic acid[238-240] and saponins isolated from other plants[241] have been shown to have anticancer activity. Dietary saponins were found to

enhance natural killer cell activity[242], presumably with an initial effect on the mucosal immune system[243]. Saponins were also found to be cytotoxic to sarcoma 37 cells[244], inhibit DNA synthesis in tumour cells[245], and decrease the growth of both human epidermal[246] and cervical[247] carcinoma cells. Konoshima and co-workers[248] have found that afromosin and soyasaponin I isolated from *Wistaria brachybotrys* exhibited remarkable inhibitory effects on mouse skin tumour promotion.

Amongst other properties ascribed to saponins, it is worth mentioning anti-oxidative[249] and estrogenic activities[250,251] and a preventive effect of liver injury[252].

1.2.10 2,3-Dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) saponins

1.2.10.1 *Nature, occurrence and chemical structures*

A new triterpenoid saponin, which is a conjugate of a γ-pyrone and soyasaponin I with an absorption maximum at 295 nm, was isolated from *Pisum sativum*[253]. The name chromosaponin I (Figure 1.15) was proposed for this new saponin. Chromosaponin I yielded soyasaponin I during extraction, but the latter was not found in the free form in this plant. In seven-day-old etiolated pea seedlings, chromosaponin I was found in all parts of seedlings, but at higher concentrations in the hook and root tip than in other non-growth tissues. Massiot *et al.*[254] isolated a new *O*-22 maltol conjugate of soyasaponin I from alfalfa seeds and soyabean and although the new compound had the same characteristics as chromosaponin I, the name of soyasaponin VI (Figure 1.15) was proposed for it. According to these authors, soyasaponin VI was an unstable saponin and might be the natural precursor of soyasaponin I. Also in 1992, Kudou and coworkers[255] isolated a new unstable saponin from soyabean seeds with a maximum absorption at 292 nm, and the structure was characterized as 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) attaching through an acetal linkage to the C-22 hydroxyl of the

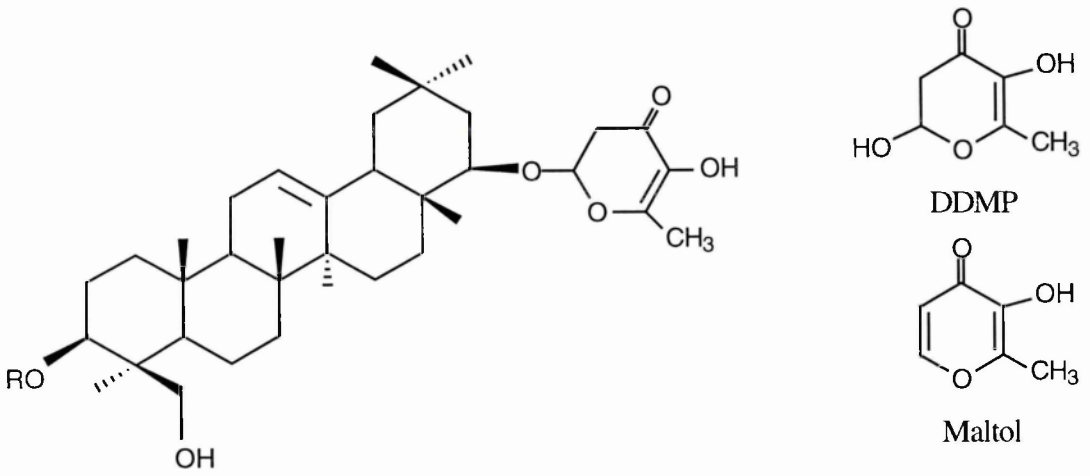


FIGURE 1.15. Structure of DDMP-conjugated soyasaponins.

Name	*R*
Soyasaponin αg	Glc(1-2)gal(1-2)glcUA-
Soyasaponin βg*	Rham(1-2)gal(1-2)glcUA-
Soyasaponin βa	Rham(1-2)ara(1-2)glcUA-
Soyasaponin γg	Gal(1-2)glcUA-
Soyasaponin γa	Ara(1-2)glcUA-

*Also called soyasaponin BeA, soyasaponin VI or chromosaponin I

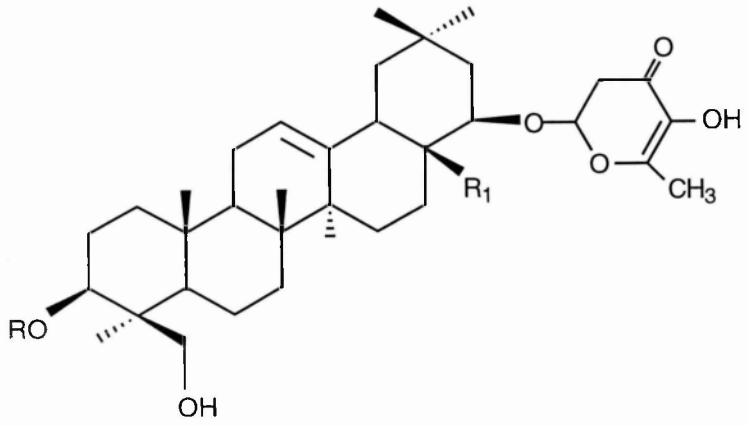


FIGURE 1.16. Structures of lablab saponin I and saponin αa.

Name	*R*	*R_1*
Lablab saponin I	Rham(1-2)gal(1-2)glcUA-	CHO
saponin αa	Glc(1-2)ara(1-2)glcUA-	CH_3

aglycone of soyasaponin I. Again, a new name, soyasaponin BeA (Figure 1.15), was proposed for this saponin, which is similar to both chromosaponin I and soyasaponin VI.

Kudou *et al.*[256] reinvestigated the composition and the structures of native 'group B saponin' in soyabean seeds. Five kinds of saponins named soyasaponins αg, βg (also called chromosaponin I, soyasaponin VI or soyasaponin BeA, as mentioned above), βa, γg, and γa (Figure 1.15), according to elution order during HPLC, were isolated and the structures were characterized as having a DDMP moiety attached via an acetal linkage to the C-22 hydroxyl of the aglycones of soyasaponins V, I, II, III, and IV, respectively, by UV, IR and NMR spectroscopy, and mass spectrometry. DDMP-conjugated saponins were detected as major saponin constituents by extraction under mild conditions, and soyasaponins I-V were not detected. Therefore it was strongly suggested that these DDMP-conjugated saponins were genuine saponins in the intact soyabeans.

A new DDMP-conjugated saponin, named soyasaponin αa (Figure 1.16), was isolated from the hypocotyl of scarlet runner bean (*Phaseolus coccineus*) together with soyasaponins αg and βg[257]. Okubo *et al.*[258] also isolated soyasaponin βg from rootstock of the American groundnut (*Apios americana*). The distribution of this DDMP-saponin in the rootstock was detected as the brown colour produced by the reaction with $FeCl_3$. A high concentration of DDMP-saponin was observed around the cells in fibrovascular bundle connecting the stem to plumule. More recently, Yoshiki *et al.*[259] have isolated a new DDMP-conjugated saponin, lablab saponin I (Figure 1.16) from the hypocotyl of hyacinth bean (*Dolichos lablab*).

Since it has been reported that many kinds of legumes such as the tubers of the Indian potato (*Apios tuberosa* Moench), the whole seeds of chickpea (*Cicer arietinum* L.), scarlet runner bean (*Phaseolus coccineus* L.), kidney bean (*Phaseolus vulgaris* L.), pea (*Pisum sativum* L.), mung bean [*Vigna mungo* (L.) Hepper], and cowpea [*Vigna sinensis* (L.) Hassk] as well as soyabean [*Glycine max* (L.) Merr.] and wild soyabean (*Glycine soja* Sieb. & Zucc.) contained DDMP saponins[260], group B saponins might be widely distributed in the legumes as DDMP-conjugated forms. However, DDMP

saponins could not be detected in the whole seeds of the cluster bean [*Cyamopsis tetragonoloba* (L.) Trub.], broad bean (*Vicia faba* L.) or azuki bean [*Vigna angularis* (Willd.) Ohwi & Ohashi]. Yoshiki *et al.*[257] also found DDMP saponins, especially soyasaponin βg, to be widely distributed in many leguminous seeds. Cowpea (*Vigna sinensis*) and mung bean (*Vigna mungo*) contained the same DDMP saponins (αg, αa and βg) as scarlet runner bean (*Phaseolus coccineus*). The existence of DDMP saponins in other leguminous seeds such as azuki beans (*Vigna angularis*) was also confirmed. Furthermore, in almost all leguminous seeds, DDMP saponins were observed solely in the hypocotyl. Nevertheless, in soyabean they were found in both the hypocotyl and the cotyledon, but the composition of the DDMP saponins was different between hypocotyl (αg, βg and/or βa) and cotyledon (βg, βa, γg and γa).

1.2.10.2 *Extraction, separation and structural elucidation*

DDMP soyasaponins were extracted from the milled seeds with 70% aqueous ethanol containing 0.01% EDTA at room temperature. The extract was evaporated to dryness under reduced pressure below 40°C and dissolved in 1 ml of water and 1 ml of 1-butanol. The butanol layer was then analyzed by HPLC[256-259].

According to Kudou and co-workers[256], soyasaponins I-V, which had been reported as major saponin constituents in soyabean, were not detected in the butanol solution, but heating of the butanol solution at 100°C for 1 h led to conversion of soyasaponins αg, βg, βa, γg and γa into soyasaponins V, I, II, III, and IV, respectively. Heating DDMP conjugated saponins changed the solution to a brown colour and produced a compound that was identified as a maltol (Figure 1.15) by HPLC and UV spectrum, which had a maximum absorption at 274 nm, compared to an authentic sample. It has been reported that the maltol is formed during cooking of soyabeans and contributes to the sweet aroma of soyabean products[261].

Soyasaponin βg has been reported [258] to be changed into soyasaponin Bb (Figure 1.5) and Be (Figure 1.6) in the presence of Fe^{3+}. HPLC analysis indicated that 2.5 mM

$FeCl_3$ rapidly changed soyasaponin βg (1 mM) into soyasaponin Bb (0.57 mM) and Be (0.43 mM), the decomposed ratio being approximately 3:2. Therefore, it is necessary to add a Fe^{3+} scavenger, such as EDTA, to the extraction solution of DDMP saponins in order to avoid decomposition. Okubo and Yoshiki[262] also found that DDMP saponins were stable in acid solution, but easily hydrolysed in alkaline solution into group B saponins and DDMP derivatives, which affect the flavour of soyabean foods.

HPLC separation of DDMP saponins was performed on a reversed-phase silica column using an EDTA-acetonitrile-water-acetic acid mixture as the mobile phase[257-259]. Detection was by UV absorption at 292 nm, which is the maximum absorption wavelength for these compounds due to the DDMP moiety.

Kudou *et al.*[256] isolated DDMP saponins from soyabean seeds using a C_{18} solid-phase column with aqueous methanol. Further purification was done by preparative HPLC using acetonitrile-water-acetic acid as the mobile phase. DDMP saponins were isolated from the hypocotyls of scarlet runner bean[257] and hyacinth[258] with aqueous ethanol containing EDTA. The extract was evaporated and dissolved in water-butanol. The butanol layer was evaporated. The crude DDMP-saponin fraction was loaded on to a Lobar column using EDTA-acetonitrile-water-acetic acid as mobile phase. The eluate was further purified by Lobar column and EDTA was removed using acetonitrile-water-acetic acid as mobile phase.

The relative molecular masses of soyasaponins αg, βg, βa, γg and γa were determined by high resolution FAB-MS, indicating the conjugation of a $C_6H_6O_3$ fragment and the corresponding soyasaponins V, I, II, III and IV. None of the DDMP saponins gave a fragment ion due to the loss of DDMP. The assignments of soyasaponins αg, βg, βa, γg and γa were established from ^{13}C- and ^{1}H-NMR spectra[263]. The structures of DDMP saponins from *Phaseolus coccineus*[257], *Apios americana*[258] and *Dolichos lablab*[259] were similarly elucidated using ^{13}C- and ^{1}H-NMR spectroscopy and chemical techniques.

1.2.10.3 *DDMP saponin content*

Although Okubo and co-workers[256-259] have successfully separated, on a qualitative basis using HPLC, intact saponins from soyabean, runner bean, American groundnut and hyacinth bean, DDMP-conjugated saponins have only been analyzed quantitatively by HPLC to a very limited extent.

Tsukamoto *et al.*[260] have examined the saponin content of the seed hypocotyl part of 414 cultivated and 149 wild soyabeans. On average, the wild soyabeans contained a larger quantity of saponins than the cultivated ones. One cultivar, however, Nattoshoryu, contained about 6% DDMP saponins on a dry weight basis. The Nattoshoryu strain maintained a high saponin content for three seasons of cultivation and is a special variety used for "natto", a traditional soyabean food in Japan produced by fermentation with the microorganism *Bacillus natto*. These authors[264] also found that the DDMP-conjugated saponin content of soyabean seeds remained stable in response to elevated temperatures during seed development. DDMP-conjugated saponin contents depended only on the variety.

Kim and Okubo[265] analyzed the saponin content of 220 cultivars of soyabean with black, yellow, green, brown and mixed testa colours, grown in different regions of Southern Korea. DDMP saponin content was found to be dependent on both the region where grown and testa colour of the soyabeans with yellow-coated soyabeans grown in both Southern and Western seashores and plain fields showing the highest DDMP saponin content.

1.2.10.4 *Properties*

As mentioned above, in seven-day-old etiolated pea seedlings, chromosaponin I was found in all parts of seedlings, but at higher concentrations in the hook and root tip than in other non growth tissues[253]. Yoshiki *et al.*[257,259] also found DDMP saponins to be mainly concentrated in the hypocotyl of leguminous seeds and according to Okubo *et*

al.[258], a high concentration of soyasaponin βg was detected in the plumule and fibrovascular bundle of American groundnut. These results imply that DDMP-saponins might play an important physiological role in the seed or plumule, possibly related to germination, or to maturation and growth.

Yoshiki *et al.*[259] have investigated the superoxide dismutase (SOD)-like activity of DDMP saponins and related substances. The SOD-like activity of the lablab saponin I (Figure 1.16) was greater than that of glutathione. The SOD-like activity of soyasaponin βg (Figure 1.15) was caused by the DDMP moiety because soyasaponin I, which does not contain a DDMP moiety, did not exhibit this activity. The difference in structure between lablab saponin I and soyasaponin βg is confined to the C-28 moiety of the aglycone, an aldehyde group in the former and a methyl group in the latter. The higher SOD-like activity of lablab saponin I suggested the radical reaction occurred not only with the DDMP moiety but that it also involved the vicinal carbon of the aglycone, especially the C-22 grouping. In the case of lablab saponin I, it is hypothesized that the C-28 aldehyde moiety exerted an effect on the C-22 group, thus promoting the radical reaction. More recently, these authors[266] found DDMP saponins to scavenge oxygen radicals when assayed using the xanthine oxidase-NH_2OH method, electron spin resonance (ESR), and chemiluminescence. DDMP saponin at a level of 1mg/ml scavenged superoxide at a degree equivalent to 17.1 units of superoxide dismutase/ml by the ESR spin trapping method. This scavenging activity of DDMP saponin is caused by the DDMP moiety attached to the triterpene aglycone. In the organism, oxygen radicals have been implicated in the initiation and development of various diseases, often mediated through chain reactions of lipid peroxidation and DNA- and protein-binding and degeneration[267,268]. These results suggest that DDMP saponins may prevent biomolecular damage due to radical attack.

1.3 OBJECTIVES

This work focused on the study of saponins in legumes of importance to both human and animal nutrition, and in particular, lupin, lentil and chickpea.

Soyasaponin I and other saponins containing soyasapogenol A as their aglycone (see Figures 1.5 and 1.6) have been detected in different species of sweet and bitter lupin whereas no saponin has been detected in the species *Lupinus albus* (see Section 1.2.3.2.a) and it has already been mentioned that environmental and agronomic conditions can be important factors affecting the saponin content of plants (Section 1.2.6.1). However, very little is known about the saponin content of lupin and the influence of such environmental and agronomic factors on the saponin content of lupin, which is a high-protein animal feed and offers a broad range of possibilities for use in human food systems (Section 1.1.2). Therefore, the first aims of this project (Chapter II) were:

- to examine differences in saponin content of 45 samples of lupin seed from five cultivars of the species *Lupinus angustifolius* and one cultivar of *L. albus*, obtained from crops grown over 2 years in 4 regions of Australia and on up to 15 sites within those regions, and
- to relate those differences in saponin content to cultivar and to agronomic variables such as growing location and year of cultivation of the lupin seed.

This type of information is useful in order to identify particular species and cultivars of lupin seed for certain properties attributed to the presence of saponins and to elucidate the possible influence of environmental and agronomic factors on the saponin content of lupin seed.

Similarly, there is little information on the level of saponins in lentils (see Section 1.2.3.1.a), which are considered to be one of the most nutritious pulses and have made a significant contribution to the human diet since ancient times (Section 1.1.3). Since there is also a lack of knowledge about the relationship between morphological seed

characteristics and saponin content of the legume seeds, the objectives of Chapter III were:

- to examine differences in saponin content of 20 cultivars of lentil seed, grown over 5 years in 18 provinces of Spain, and
- to relate such differences in saponin content to size and testa colour of the lentil seeds.

This information is relevant in the screening and selection of low or high saponin-containing cultivars of lentil as part of any future breeding programme with the aim of improving the compositional quality of this type of legume seeds.

Another aim of this project was:

- to isolate possible new saponins from any of the studied legume seeds (Chapter IV) in sufficient quantity to enable their structure to be elucidated.

On the other hand, it has already been mentioned (see Section 1.2.5) that a number of chromatographic methods have been used for saponin analysis and particular attention has focused on the use of gas chromatography (GC) (Section 1.2.5.3) but it can only be used for the separation and quantification of the aglycone portion of the saponin. Therefore, the objectives of Chapter V were:

- to develop a quantitative analysis of individual saponins from lupin seed (*L. angustifolius*) using an improved high-performance liquid chromatographic (HPLC) method based on one used previously for the qualitative determination of saponins, and
- to compare the results obtained by HPLC with those using the conventional GC method for the analysis of saponins.

The developed technique enables quantitative studies to be made for the first time on the effect of processing not only on the total saponin content but also on the true saponin composition, which, in turn, allows the fate of individual saponins to be determined. Since some of the biological properties reported for saponins are now recognised to be dependent on their particular chemical structures (Sections 1.2.9.1 and 1.2.10.4), this type of information allows further understanding of not only the

relationship between chemical structure and bioactivity but also the impact of processing on that bioactivity in relation to both human and animal health.

For those reasons, once the objectives mentioned above had been achieved, the method developed was applied to the study of different kinds of processing on the saponin content and composition of lentils and chickpeas, which, as for lentils, are used mainly for human consumption and are thought to contain the highest protein quality of all the pulses (Section 1.1.4). In other words, the latter part of this work (Chapter VI) involved a study of:

- the effect of soaking using distilled water, citric acid and bicarbonate solutions,
- the effect of cooking for 30, 60, 90 and 120 min, after the seeds were presoaked in distilled water, and
- the effect of germination in the dark for 6 days at 20°C on both the saponin content and the composition of two cultivars of both chickpeas and lentils.

II Effect of Cultivar and Environment on Saponin Content of Australian Sweet Lupin Seed

2.1 INTRODUCTION

Lupin is a leguminous crop which has been used as a human food by ancient cultures surrounding the Mediterranean and by people living in the Andean highlands (see section 1.1.2.2). Both *Lupinus angustifolius* and *L. albus* are, probably, the most important species of *Lupinus*. While the former (the narrow-leaved or blue lupin), which originated in coastal areas bordering the Mediterranean, is now of commercial importance in Australia and New Zealand, the latter (the white or Egyptian lupin) is the oldest established cultivated species among the Mediterranean lupins and is also currently cultivated in Central Europe, South Africa, South America and Australia[10].

Although the advantages of lupin as green fodder, silage, high-protein animal feed or as a soil improver have been recognised, the use of lupin seeds in human nutrition has not expanded. In Australia, for example, lupin flour and hull fibre have a limited use in bread manufacture and through retail "Health Store" outlets[7].

One of the main reasons that lupin has limited use in human consumption is the high level of alkaloids which historically influenced the taste and digestion of lupin seed. Nevertheless, the levels of alkaloids in modern varieties of sweet lupin are considered safe and antinutritional factors are also found at a low level (see section 1.1.2.3). However, very little is known about the saponin content of lupin, which might also interfere with the nutritional use and acceptance of lupin in human diets. There is also a lack of information about the factors which may affect the saponin content of lupin.

Soyasaponins have been detected in different species of sweet and bitter lupin but no saponin was detected in *L. albus* [79,80]. The near 10-fold difference observed between the varieties containing saponins studied by Muzquiz *et al.*[79] indicates the scope for manipulating the level of this class of compounds by selective breeding. On the other hand, Cuadrado *et al.*[80] postulated that the environmental conditions of the Peruvian highlands were important factors in determining the amount of saponins present in lupin. Nevertheless, several studies carried out in soyabeans[161,260,264] revealed that the

saponin content of the seeds was more strongly dependent on the variety than on environmental effects.

The aim of this work was to examine differences in saponin content of 45 samples of lupin seed grown in Australia and relate those differences to both cultivar and agronomic variables. Such information will be useful for identifying particular varieties and cultivars of lupin for properties such as undesirable taste or beneficial physiological activity which can be attributed to the presence of saponins (see section 1.2.9).

2.2 MATERIALS

Forty-five samples of lupin seed from six cultivars of sweet *L. angustifolius* (Danja, Gungurru, Yorrel, Warrah, Illyarrie, Merrit) and one cultivar of sweet *L. albus* (Kiev mutant) (Figure 2.1) were obtained from crops grown in 1990 and 1991 in areas of Western Australia, Victoria, New South Wales and South Australia and on up to 15 sites within those areas.

A standard sample of soyasaponin VI, also called soyasaponin ßg, was kindly donated by Dr. K. Okubo and Dr. Y. Yoshiki (Tohoku University, Japan). Soyasaponin I was available from previous work[95].

All solvents used during the extraction process were of analytical grade and redistilled before use. Chloroform, methanol, ethyl acetate, acetic acid, sulphuric acid, *p*-anisaldehyde, ammonium hydroxide, phosphorus pentoxide and anhydrous sodium sulfate were purchased from BDH Laboratory Supplies, Poole, U.K. Acetyl chloride and cholesteryl-n-decanoate were supplied by Sigma Chemical Co., Poole, Dorset, UK. Both bis(trimethylsilyl) trifluoroacetamide and pyridine of silylation grade were provided by Pierce, Rockford, Illinois, U.S.A. Water was purified using a Milli-Q system, Waters Chromatography Division, Millipore Corporation, Milford, U.S.A.

Reversed- and normal phase thin layer chromatography plates were purchased

from Whatman International Ltd., Maidstone, U.K. and E. Merck, Darmstadt, Germany, respectively.

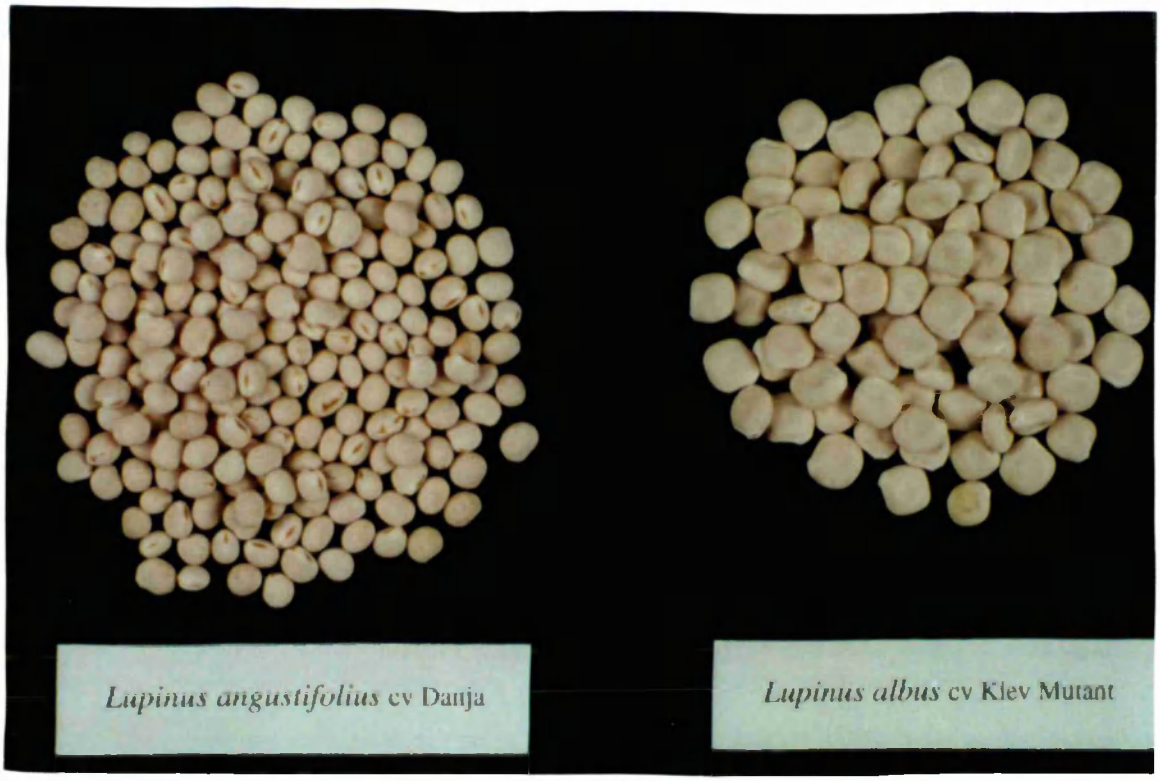


FIGURE 2.1. Seed of *L. angustifolius* and *L. albus*.

2.3 METHODS

The method used for the analysis of saponins was based on the one developed by Price *et al.*[50].

This method includes a hot solvent extraction of saponins, after the seed flour has been defatted. The saponin extract is then partially purified and acid hydrolysed to produce a sapogenol, which after derivatisation, is separated and quantified using gas chromatography (Figure 2.2). The relative molecular masses of the saponins in the original extract are estimated using fast atom bombardment mass spectrometry and the saponin content of the seed is calculated from these data.

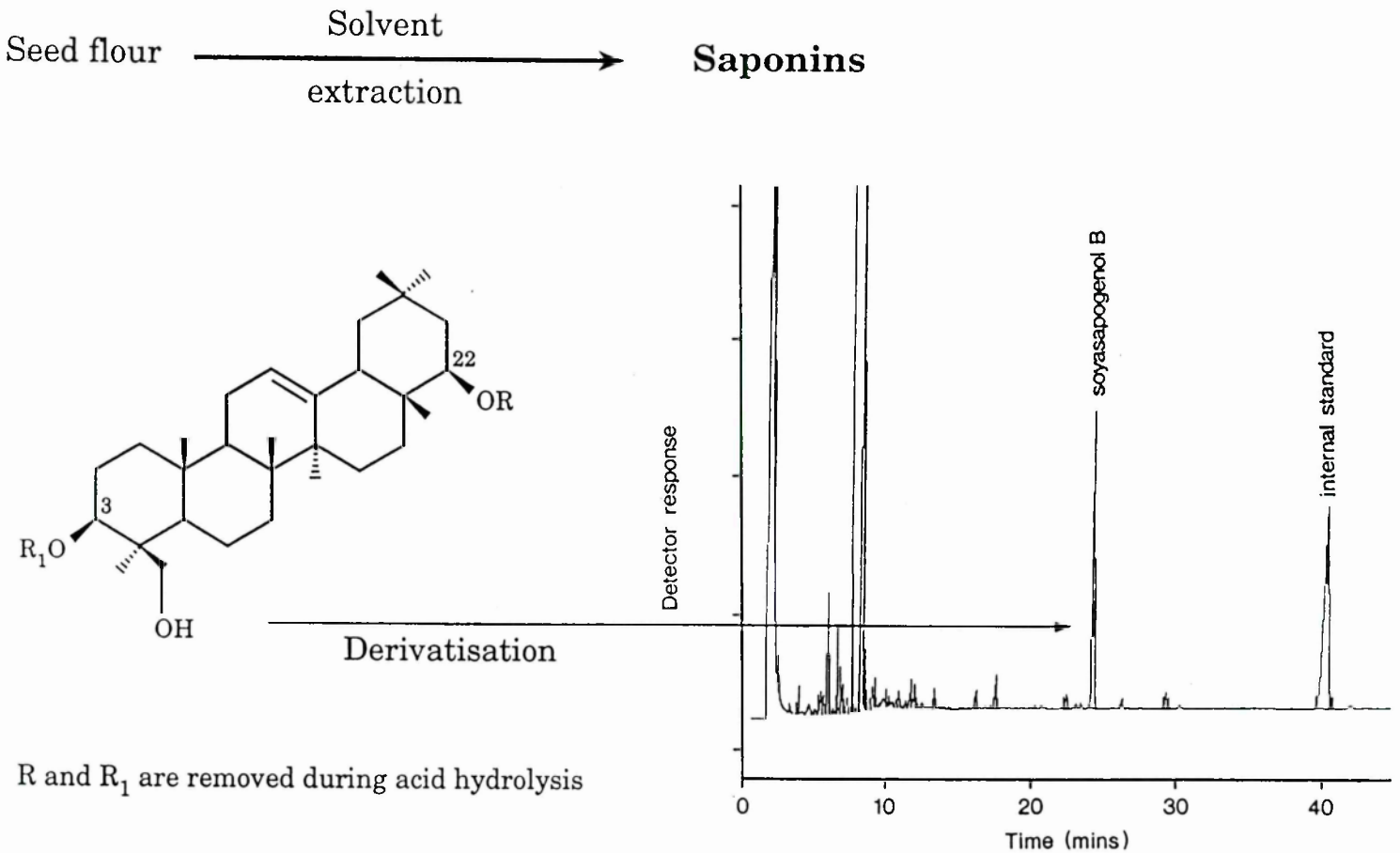


FIGURE 2.2. Analysis of saponins using a gas chromatographic method[50].

2.3.1 Extraction of saponins

The ground sample (5 g) was mixed with sand (10 g) and extracted in a Soxhlet apparatus sequentially with chloroform (200 ml, 16 h) and methanol (200 ml, 30 h). The methanol extract was evaporated to dryness *in vacuo* and redissolved in water (5 ml). The aqueous solution was eluted through a glass column dry-packed with SiO_2-C_{18} (20 g; J T Baker UK, Hayes, Middlesex, UK) which had previously been conditioned first with methanol (100 ml) and then with water (150 ml). The elution was carried out in two stages, firstly with water (150 ml) and then with methanol (150 ml) under a pressure of 10 psi (1 psi ≈ 6.9 kPa). The methanol fraction was evaporated to dryness under reduced pressure and the residue made up to 5 ml in methanol, corresponding to 1 g ml^{-1} of the starting material, ready for acid hydrolysis.

2.3.2 Fast atom bombardment-mass spectrometry (FAB-MS)

Aliquots of 5 µl of each methanol solution were added to a drop of glycerol on the FAB copper probe tip. Mass spectra were obtained using a Kratos MS 9/50TC mass spectrometer by bombardment of the sample with an atom beam of xenon produced by an Ion-Tech 11 NF atom gun operating at 9 kV (nominal). Positive and negative ion spectra were recorded using a UV galvanometer recorder.

2.3.3 Thin-layer chromatography of saponins

Aliquots of 10 µl of the methanol solution were spotted onto plates and developed in two systems: (a) C_{18} bonded to silica gel (Whatman KC_{18}; 20 x 20 cm; layer thickness: 0.25 mm); methanol:water (3:2); (b) silica gel 60 (Merck silica gel 60; 20 x 20 cm; layer

thickness: 0.20 mm); chloroform:methanol:water (65:35:10, lower phase). Both plates were developed for a distance of 12 cm, air-dried and sprayed with *p*-anisaldehyde/glacial acetic acid/ 97% w/v sulphuric acid (1:100:2). Spots were visualized by gentle heating with hot air for 5 min.

2.3.4 Acid hydrolysis of saponin fractions

Two aliquots (1 ml) of each of the methanol solutions were evaporated to dryness and dried over phosphorus pentoxide in a vacuum desiccator overnight. The residues were refluxed for 3 h with acetyl chloride in methanol (5% v/v, 5 ml, a source of dry HCl). After cooling, the hydrolysates were neutralised with aqueous ammonium hydroxide (2M) and evaporated to dryness. After redissolving in water (5 ml), the sapogenols were extracted with ethyl acetate (3 x 5 ml). The first 5 ml of ethyl acetate contained a standard of cholesteryl-n-decanoate (0.2 mg ml^{-1}). The combined ethyl acetate extracts were dried over anhydrous sodium sulfate, filtered and evaporated to dryness.

2.3.5 Gas chromatography of sapogenols

The residues equivalent to 1 g of defatted flour were redissolved in ethyl acetate (1 ml) and evaporated to dryness in a vial by a stream of nitrogen. After vacuum drying over phosphorus pentoxide for 12 h, the hydrolysate was derivatised by heating with bis(trimethylsilyl) trifluoroacetamide (100 µl) and pyridine (100 µl) in sealed vials (50°C, 20 min). Five microlitres of each sample were injected onto a Hewlett Packard 5890 Series II gas chromatograph fitted with a glass column (30 m x 0.32 m id) coated with DB-1, 0.25 micron film (J&W Scientific, Serial No 1129125) and flame ionisation detector. The maximum oven temperature was 340°C, detector 300°C and injector 290°C,

with helium as the carrier gas.

2.3.6 Gas chromatography-mass spectrometry (GC-MS) of sapogenols

Peak identity of the derivatised sapogenols was determined by coelution with standards as well as by GC-MS. The column was the same as used for gas chromatographic estimation of sapogenols above, and was directly coupled to a TRIO-1S mass spectrometer (Fisons Instruments), operating at a source temperature of 250°C and a nominal ionising voltage of 70 eV.

2.3.7 Quantitative determination of saponins and sapogenols

Quantification was achieved using genuine soyasaponin I isolated from pea seed[95], against an internal standard of cholesteryl-n-decanoate. The relationship between the weight of soyasaponin I and the peak area ratio of soyasapogenol B with internal standard was a straight line with a correlation coefficient of 0.995.

2.3.8 Statistical methods

The data were subjected to standard two-way analyses of variance (without replicates), using Minitab 8.21 Software (Macintosh version), to determine the statistical significance of differences between the sources of variation being examined; a random model[269] was assumed throughout. Percentage contributions of the sources of variation to the total variation were calculated using the expected mean squares of the random model, these are represented by 0 in cases where the calculations give negative estimates. It should be

noted that in the absence of replication at each site, no suitable error is available to test the genotype x environment interaction represented by the cultivar x regions, sites and years items in the tables; however, the importance of the interaction relative to the other sources of variation can be seen from the percentage contributions.

2.4 RESULTS*

2.4.1 Saponin composition

Saponins were detected in all the lupin seed of *Lupinus angustifolius* but not in *L. albus* seed.

The FAB mass spectra for the *L. angustifolius* extracts containing the intact saponins, after exhaustive hot methanol extraction, showed the presence of 3 saponins with relative molecular masses of 942, 1068 and 1104 respectively. The common aglycone of these was confirmed to be soyasapogenol B (Figure 1.5; see section 1.2.3.1.a) by GC-MS.

From their thin layer chromatographic behaviour, the first saponin was identified as soyasaponin I (Figure 2.3) and the second as soyasaponin VI (Figure 2.3). R_f values for soyasaponins I and VI were 0.16 and 0.08, and 0.29 and 0.37 in reversed- and normal phase TLC, respectively. Soyasaponin VI[254], also known as soyasaponin ßg[256], contains 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) at the C-22 position of soyasaponin I. The third saponin appeared to be novel. R_f values for this new saponin in normal and reversed-phase TLC plates were 0.22 and 0.60, respectively.

* The data in this chapter have been published in:

Ruiz, R.G., Price, K.R., Rose, M.E., Arthur, A.E., Petterson, D.S. and Fenwick, G.R. (1995). *J. Sci. Food Agric.*, **69**: 347-351.

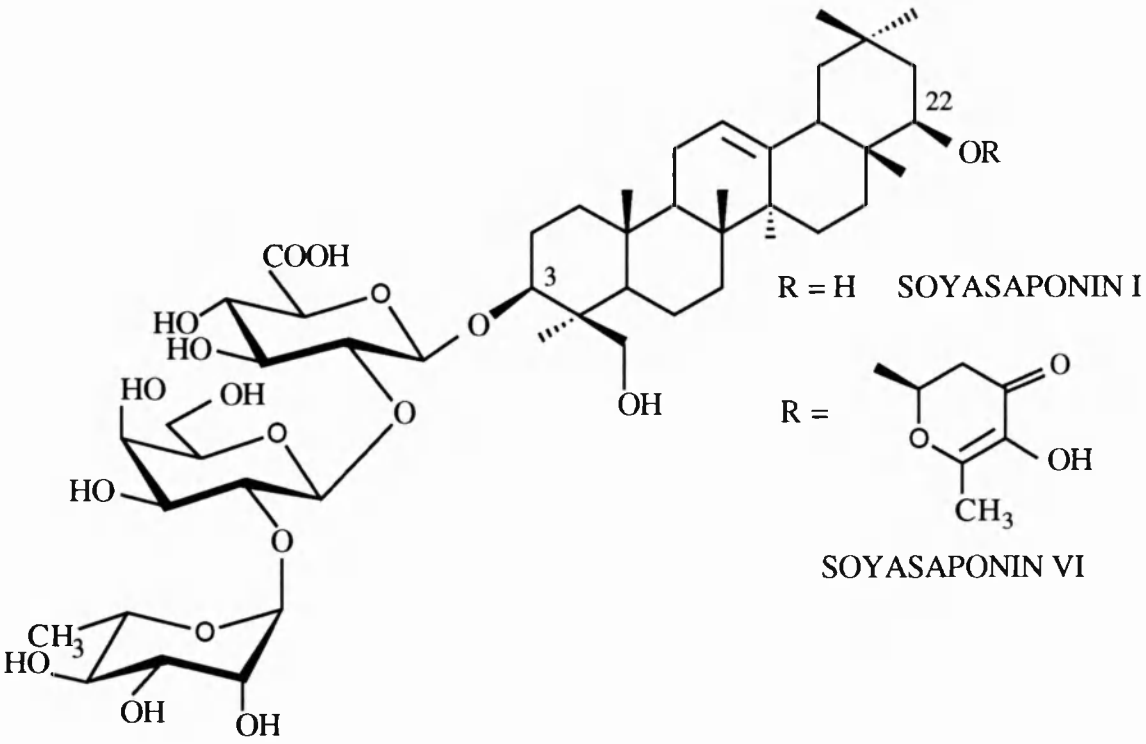


FIGURE 2.3. Chemical structures of soyasaponin I and soyasaponin VI.

Soyasaponins I and VI were calculated roughly to be in equimolar proportions using semiquantitative estimation based on a comparison of molecular ion peak heights from FAB mass spectra. The new saponin was present at a much lower concentration, assuming that it had a similar mass spectrometric behaviour to the other two soyasaponins.

2.4.2 Saponin content

2.4.2.1 *Effect of cultivar and region*

Total saponin content of the cultivars grown in the various regions is shown in Table 2.1 and Figures 2.4 and 2.5.

Analysis of variance for these data is shown in Table 2.2. 'Residual' is the error

term, which is the variation left over during the partitioning of the total variance into the recognisable sources of variation, i.e. the two 'between' items. Therefore, the total variance in Table 2.2, which will have 3+2+6=11 degrees of freedom (df), is partitioned into 'between regions' (3 df) and 'between cultivars' (2 df) with 6 df left over (the 'residual'), which becomes the error term in the analysis of variance, being used to test or divide into the two 'between items'. The statistical probability has been written as P (Table 2.2), followed by the level of statistical significance associated with it. Although the actual probabilities vary between 0 and 1, they are often expressed as percentages. The 5% level is usually the threshold in biology (anything less than this value is considered to represent statistical significance). This means that, with a null or basic hypothesis that there are no differences, the probability measures how similar groups are; therefore, a low probability (less than 5%) indicates that these groups are unlikely to be the same. P = 5 - 1%, P = 1 - 0.1%, P < 0.1% indicates increasing probability that groups are not the same, or increasingly different.

No large inter-regional variations in total saponin content were recorded in the 1990 growing year (Tables 2.1 and 2.2; Figure 2.4). However, important and significant differences between lupin cultivars were found. Yorrel had a consistently lower saponin content (mean value: 411 mg kg^{-1}) than the other two cultivars, Danja and Gungurru, which had similar levels of saponin (mean values: 545 and 554 mg kg^{-1}, respectively).

In 1991, there were no significant inter-regional differences for either Danja or Gungurru (Tables 2.1 and 2.2; Figure 2.5); Yorrel was not grown. Cultivar differences were also found to be significant in 1991. Danja produced a higher level of saponin in 1991 compared with 1990, while the level for Gungurru was essentially the same as that for 1990.

The highest saponin content for Gungurru was found in seed grown in Western Australia for both years while the highest levels for Danja were in seed grown in South Australia (1990) and New South Wales (1991). The cultivar Yorrel grown in South Australia (1990) contained the highest level of saponin.

TABLE 2.1 Effect of cultivar and location (region) on the total saponin content (mg kg^{-1}) of *Lupinus angustifolius*.

Year	*Cultivar*	*Region*				*Mean Value*	*Range*
		Western Australia	*Victoria*	*New South Wales*	*South Australia*		
1990	Danja	540	522	538	579	545	522 - 579
	Gungurru	611	521	575	508	554	508 - 611
	Yorrel	379	---	412	442	411	379 - 442
1991	Danja	709	---	740	626	691	626 - 740
	Gungurru	596	579	527	579	570	527 - 596

TABLE 2.2 Statistical significance of differences between regions and between cultivars.

Year	*Source of Variation*	*Degrees of Freedom*	*Mean Squares*	*% Variance Component*
1990	Between regions	3	77	0
	Between cultivars	2	21671***	71
	Residual	6	1977	29
1991	Between regions	3	2157	0
	Between cultivars	1	24090*	44
	Residual	3	2805	56

* P = 5 - 1 %; ** P = 1 - 0.1%; *** P< 0.1%

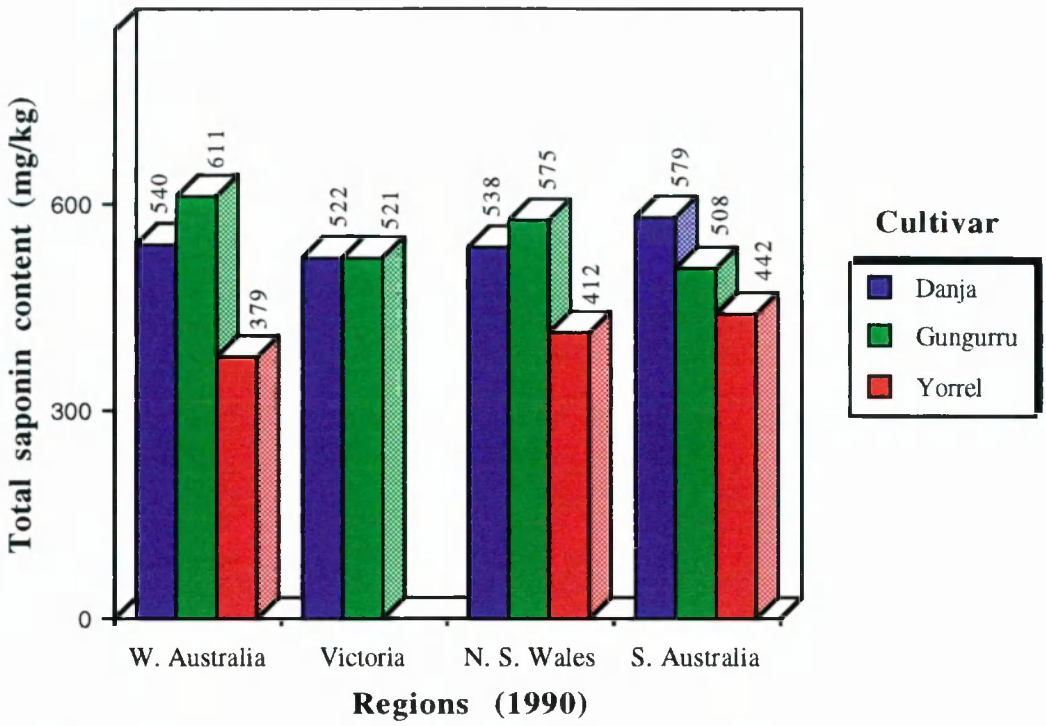


FIGURE 2.4. Effect of cultivar and location (region) on the total saponin content (mg/kg) of L. angustifolius in the 1990 growing year.

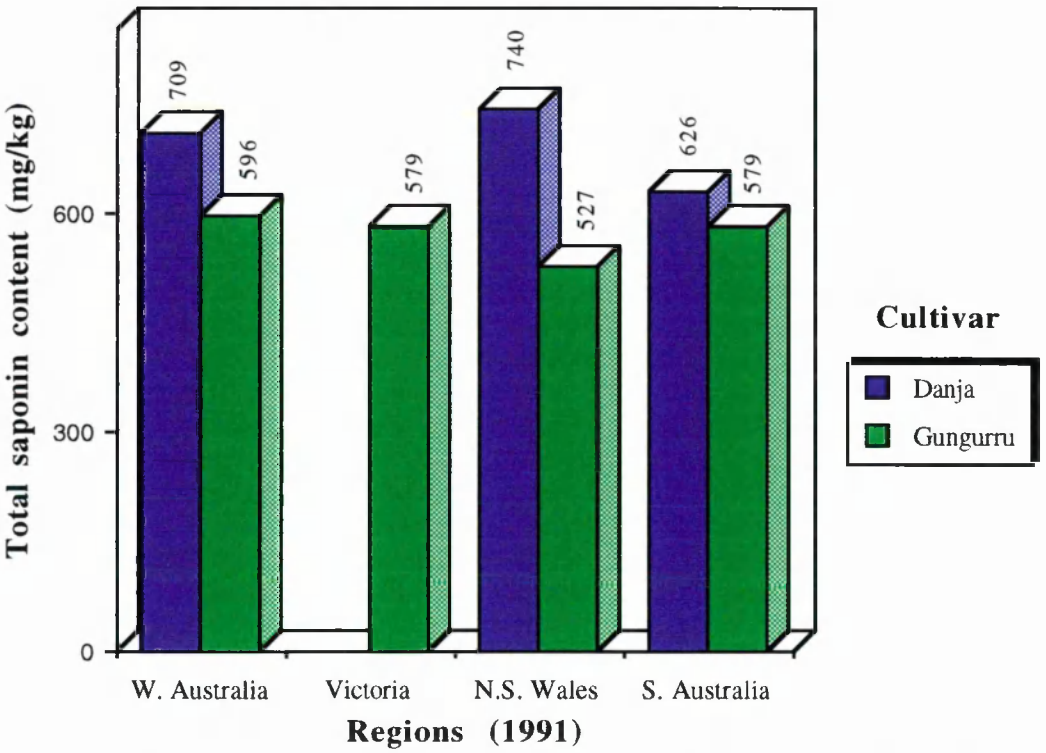


FIGURE 2.5. Effect of cultivar and location (region) on the total saponin content (mg/kg) of L. angustifolius in the 1991 growing year.

2.4.2.2 *Effect of cultivar and site within a region*

Total saponin content of the cultivars grown in the various sites within the South Australia region are shown in Table 2.3 and Figures 2.6 and 2.7. Analysis of variance for data in Table 2.3 is shown in Table 2.4.

The range of saponin contents in seed grown on sites within the South Australia region was larger than those observed between regions (Tables 2.1 and 2.3). The cultivars Warrah and Illyarrie grown in 1990 had saponin contents ranging from 516 to 731 mg kg^{-1} and from 433 to 623 mg kg^{-1}, respectively (Table 2.3; Figure 2.6). There were also significant differences between cultivars with mean values for Warrah and Illyarrie of 601 and 533 mg kg^{-1}, respectively (Tables 2.3 and 2.4). The cultivar Warrah had a higher saponin content than Illyarrie for all the sites in South Australia during 1990. Moreover, for both cultivars, Mannanarie was the site where the highest saponin content was found, followed by Kapunda, Kapinnie and Kangaroo (Table 2.3; Figure 2.6)

Similar behaviour was observed for the cultivars Warrah and Yorrel grown in South Australia during 1991 and again Mannanarie was the site where the highest saponin content was found, followed by Kybybolite, Struan and Kapinnie (Table 2.3; Figure 2.7) . However, seed harvested in 1991 (Tables 2.3 and 2.4) did not show such large differences between sites (Warrah, 558-672 mg kg^{-1}; Yorrel, 404-517 mg kg^{-1}), although there were significant differences between cultivars (mean values for Warrah and Yorrel were 599 and 478 mg kg^{-1}, respectively) with Warrah having a larger saponin content than Yorrel for all the sites studied (Table 2.3; Figure 2.7).

TABLE 2.3 Effect of cultivar and location (site within a region) on the total saponin content (mg kg^{-1}) of *Lupinus angustifolius*.

Year	*Cultivar*	*Sites within South Australia region*						*Mean Value*	*Range*
		Kapunda	*Kangaroo*	*Mannanarie*	*Kapinnie*	*Kybybolite*	*Struan*		
1990	Warrah	637	516	731	522	---	---	601	516 - 731
	Illyarrie	610	433	623	468	---	---	533	433 - 623
1991	Warrah	---	---	672	558	597	569	599	558 - 672
	Yorrel	---	---	517	404	464	454	478	404 - 517

TABLE 2.4 Statistical significance of differences between sites and between cultivars.

Year	*Source of Variation*	*Degrees of Freedom*	*Mean Squares*	*% Variance Component*
1990	Between sites	3	19317***	77
	Between cultivars	1	9180**	18
	Residual	3	631	5
1991	Between sites	3	4601**	18
	Between cultivars	1	38781***	80
	Residual	3	182	2

* P = 5 - 1 %; ** P = 1 - 0.1%; *** P< 0.1%

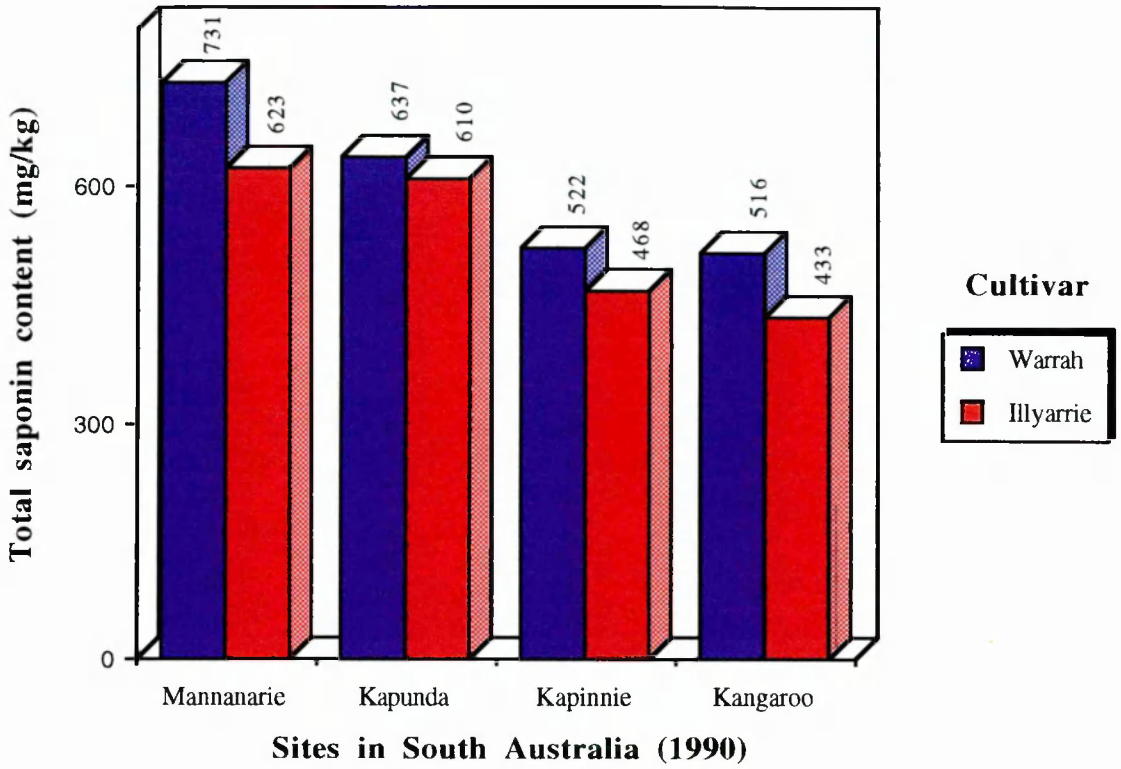


FIGURE 2.6. Effect of cultivar and location (site within a region) on the total saponin content (mg/kg) of L. angustifolius in the 1990 growing year.

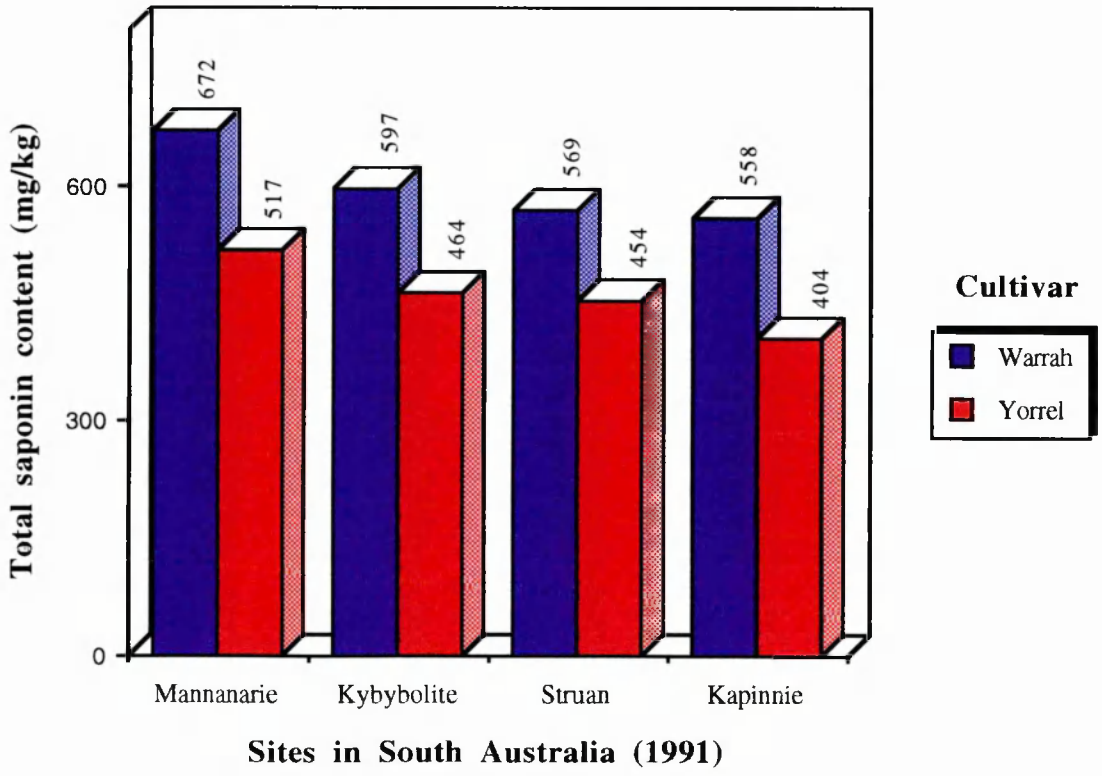


FIGURE 2.7. Effect of cultivar and location (site within a region) on the total saponin content (mg/kg) of L. angustifolius in the 1991 growing year.

2.4.2.3 *Effect of cultivar and growing year*

Table 2.5 and Figure 2.8 show the year to year variation of total saponin content of 4 cultivars of *L. angustifolius* grown in the same two sites in South Australia during 1990 and 1991.

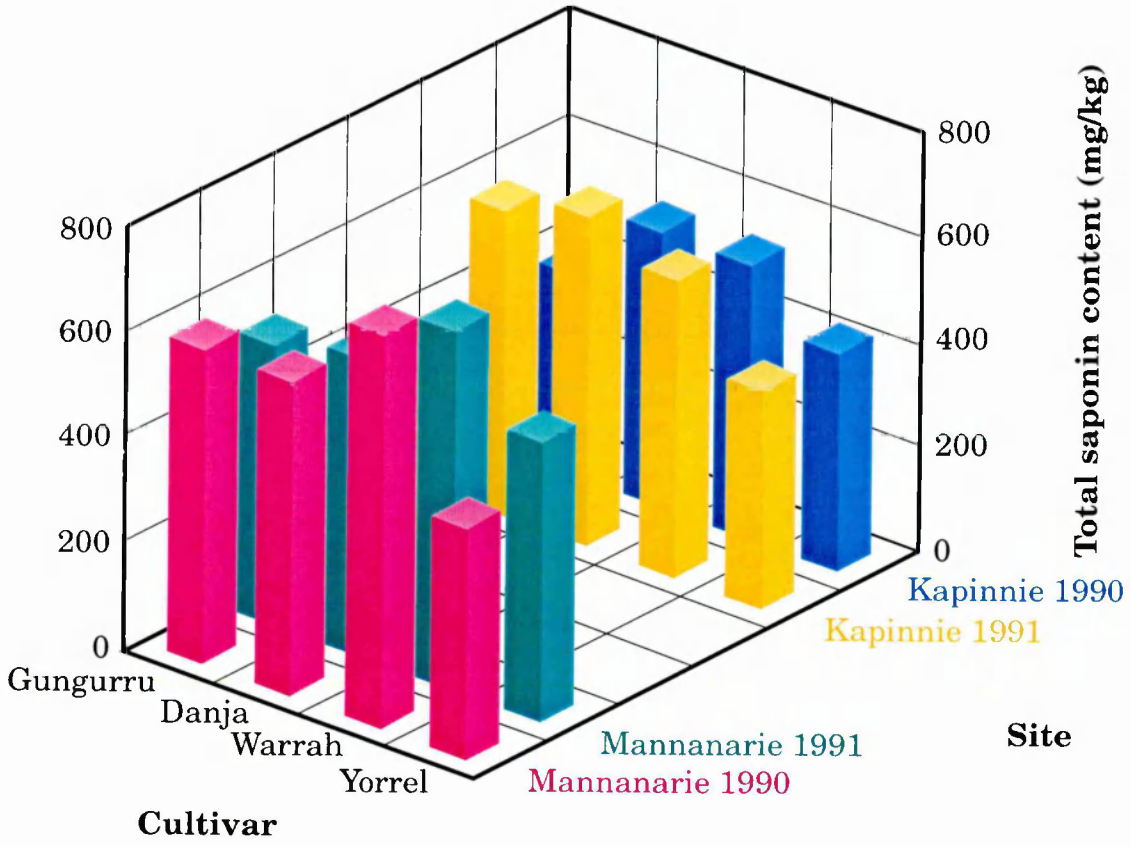


FIGURE 2.8. Effect of cultivar, site and growing year on the total saponin content (mg kg^{-1}) of *Lupinus angustifolius*.

Analysis of variance for data in Table 2.5 is shown in Table 2.6. In an experiment where there are interactions, such as in Table 2.6, it is usual to test the interactions first by dividing their mean squares by the residual mean square. If none of these interactions are significant, the degrees of freedom (df) for the residual mean square can be increased (in order to provide a better test) by 'pooling' (adding together) the non significant interaction df with the residual df. This is done simply as 3+3+1+3 in Table 2.6 (for the three interactions df and the residual df) to give 10 df for the 'Pooled residual'. The interaction sums of squares are similarly pooled with the residual sums of squares to provide a new 'Pooled sums of squares'. A 'Pooled mean square' is then derived by dividing the 'Pooled sums of squares' by the 'Pooled df' and this is used to test (divide into) the 'Between items'. The increase in the degrees of freedom for the 'Pooled mean square' simply increases the accuracy of the test.

Significant differences were found between the cultivars and between the sites, but not between the years of cultivation. It is interesting to note that all the interaction items in the analysis - cultivar x year, cultivar x site and year x site - were all statistically non-significant indicating that, on average, the rank orders for saponin content were relatively consistent over cultivars, years and sites (Table 2.6).

TABLE 2.5 Effect of cultivar, site and growing year on the total saponin content (mg kg^{-1}) of *Lupinus angustifolius*.

Year	*Cultivar*	*Sites within South Australia region*	
		Mannanarie	*Kapinnie*
1990	Danja	584	528
	Gungurru	587	386
	Warrah	731	522
	Yorrel	424	410
1991	Danja	561	626
	Gungurru	534	579
	Warrah	672	558
	Yorrel	517	404

TABLE 2.6 Statistical significance of differences between cultivars, between years, between sites and interactions: cultivar x year, cultivar x site and year x site.

Source of Variation	*Degrees of Freedom*	*Mean Squares*	*% Variance component*
Between cultivars	3	24423*	43.3
Between years	1	4865	0.5
Between sites	1	22276	13.2
Cultivar x year	3	1155	0
Cultivar x site	3	4648	0
Year x site	1	8236	5.8
Residual	3	5087	37.2
Pooled residual	10	4090.5	

* P = 5 - 1%; ** P = 1 - 0.1%; *** P < 0.1%

2.5 DISCUSSION

Lupinus angustifolius has been reported here to be a legume with a relatively low saponin content (range : 379 - 740 mg kg^{-1}) compared to soyabean, haricot or kidney bean which showed saponin levels of 6500, 4100 and 3500 mg kg^{-1}, respectively[50]. Saponins were not detected in *L. albus*, which confirms previous reports [79,80] on this particular variety of lupin.

The presence of two saponins with relative molecular masses of 942 and 1104 is in agreement with Muzquiz *et al.*[79], who also found saponins with the above mentioned relative molecular masses in sweet *L. angustifolius* cultivated in Spain. However, from mass spectral data there do appear to be structural differences between the saponins found in seed from the two countries. The seed grown in Spain contained saponins with the aglycones of both soyasapogenol B and soyasapogenol A (Figure 1.5; see section 1.2.3.1.a) whereas the Australian *L. angustifolius* had saponins containing only the aglycone soyasapogenol B.

The presence of soyasaponin VI, which has been isolated from alfalfa and soybean[254,256] and characterised as a possible precursor of soyasaponin I, was detected only in the seed grown in Australia[254]. This is interesting as Kudou *et al.*[255,256] have suggested that this maltol conjugate of soyasaponin I is really the genuine saponin in the intact soyabeans and has also been detected in a range of other legumes[257,260] (see section 1.2.10.1).

The results reported in this work have shown that the changes observed in total saponin content of seed from six cultivars of lupin seed from sweet *L. angustifolius* were mainly dependent on cultivar, followed by the site and then the region where grown. However, no significant effect was found between growing seasons. These results are in good agreement with those reported by Shiraiwa *et al.*[161], who observed that the content of saponin in soyabean seed was more greatly dependent on the variety

than on the cultivation year and the degree of maturity of the seed. There is also a good concordance between the findings reported here and the results described by Tsukamoto *et al.*[264], who also found that the saponin content of soyabean seeds was dependent on the variety but remained stable in response to both sowing dates and elevated temperatures during seed development.

Cuadrado *et al.*[80] found a significantly higher level of soyasapogenol A in *L. mutabilis* grown in the Peruvian highlands than the one obtained by the group[79] when the same variety of lupin, but of Spanish origin, was analysed in identical conditions. These results led these workers to conclude that the agroclimatic and altitude conditions of the Peruvian highlands were dominant factors in determining both the amount and composition of saponins present in bitter lupin.

These findings are in good agreement with the results described here due to the fact that a strong correlation was found between the total saponin content and the site where the lupin seed was grown (see section 2.4.2.2), which suggests that some specific environmental factors such as temperature, rainfall, and type of soil at a particular site could be responsible for higher levels of saponin in the lupin seed. A further study comparing changes in the environment of a particular region is needed to decide which of these factors are important.

2.6 CONCLUSIONS

- The total saponin content in 45 samples of lupin seed from sweet *L. angustifolius* ranged from 379 to 740 mg kg^{-1} whereas no saponin was detected in sweet *L. albus*.

- Soyasaponin I, soyasaponin VI and a possible new saponin, at a much lower concentration, are the saponins present in the Australian *Lupinus angustifolius*.

- The total saponin content of Australian *L. angustifolius* is largely dependent on cultivar.

- The total saponin content was also found to depend on the location where the *L. angustifolius* seed was grown; the most significant differences are shown when comparing data between sites within the same region. Furthermore, a strong correlation between total saponin content and growing site was found, which suggests that some specific environment factors at a particular site could be responsible for higher levels of saponin in the *L. angustifolius* seed.

- No significant differences were found between the years of cultivation of the *L. angustifolius* seed.

III Effect of Seed Size and Testa Colour on Saponin Content of Lentil Seed

3.1 INTRODUCTION

Lentils have made a significant contribution to the human diet since ancient times. There are two subspecies of lentils, *Lens culinaris* ssp. *macrosperma*, which is characterized by flat pods and large seeds, and *Lens culinaris* ssp. *microsperma*, which has small seeds and convex pods. The seeds show considerable variation in both testa and cotyledon colour. The subspecies *macrosperma* includes the so-called Chilean or yellow cotyledon types of lentil while the subspecies *microsperma* includes the Persian lentils or red cotyledon types.

Lentils are considered to be one of the most nutritious pulses. However, they also contain several antinutritional factors which could limit their consumption (see section 1.1.3.3). The information on the level of saponins in lentils is scanty. Price *et al.*[50] reported a saponin content for lentils of 1.1 g kg^{-1}.

The possibility of producing lentil varieties, optimised for their end use and meeting nutritional requirements, whilst reducing undesirable components, for instance, the raffinose family of alpha-galactosides producing flatulence[270], has become of increasing importance for the modern breeding programmes of this type of legume. In the case of saponins in lentils, very little is known about the relationship between these bioactive compounds and morphological seed characteristics. Fenwick and Oakenfull[126], for example, estimated saponin contents of 121, 136 and 107 g kg^{-1} for small red, large red and brown lentils, respectively, using quantitative thin layer chromatography. However, the levels measured by these authors are considerably in excess of those found by other workers using alternative methods[130,127]. Therefore, there is clearly a need to provide relevant information in order to allow selections and crosses to be made with the aim of improving the compositional quality of lentils.

The objective of this work was to examine differences in the saponin content of 20 cultivars of lentil seed grown in Spain in relation to seed size and testa colour.

3.2 MATERIALS

Seed from 20 cultivars of *Lens culinaris* Medik. (Figure 3.1) were obtained from one crop of each grown between 1987 and 1992 in 18 provinces of Spain.

Standard samples of saponins and chemicals were the same as those described in Section 2.2.

FIGURE 3.1. Seed from some of the cultivars of Spanish lentils.

3.3 METHODS

3.3.1 Saponin analysis

Extraction and analysis of saponins were carried out as described in Section 2.3.

3.3.2 Statistical methods

The data were subjected to standard one-way analyses of variance (unequal groups), using Minitab 8.21 Software (Macintosh version) to determine the statistical significance of differences between the sources of variation being examined.

3.4 RESULTS*

3.4.1 Saponin composition

Saponins were detected in all the lentil seed. The FAB mass spectra showed the presence of two saponins in the mixture with relative molecular masses of 942 and 1068. The common aglycone of these was confirmed to be soyasapogenol B (Figure 1.5; see section 1.2.3.1.a) by GC-MS. From their thin layer chromatographic behaviour, the former saponin was identified as soyasaponin I (Figure 2.3) and the latter as soyasaponin VI (Figure 2.3) (see section 2.4.1).

* The data in this chapter have been published in:

Ruiz, R.G., Price, K.R., Rose, M.E. and Fenwick, G.R. (1996). *Food Chem.* (in press).

3.4.2 Saponin content

Seed characteristics and total saponin content of the 20 cultivars of *Lens culinaris* Medik. grown in 18 different provinces of Spain are shown in Table 3.1. Saponin content in the harvested seed ranged from 654 to 1269 mg kg^{-1}.

3.4.2.1 *Effect of seed size*

Table 3.2 and Figure 3.2 show the relationship between lentil seed size and total saponin content. *Macrosperma* seeds (100 seed weight > 4.5 g) were found to have a significantly larger total saponin content (1105 mg kg^{-1}) than *microsperma* seeds (100 seed weight < 4.5 g), which showed a mean saponin level of 929 mg kg^{-1}.

3.4.2.2 *Effect of testa colour*

Table 3.3 and Figure 3.3 show the effect of seed testa colour on the total saponin content of lentils. Although no significant differences were found between seeds with beige or green testas, whose saponin levels were 1106 and 1089 mg kg^{-1}, respectively, brown-coated lentils showed a highly significant lower saponin content (800 mg kg^{-1}).

TABLE 3.1. Cultivar, growing site, 100 seed weight (g), seed cotyledon and testa colour, and total saponin content (mg kg^{-1}) of Spanish *Lens culinaris* Medik.

Cultivar	Growing site	100 seed weight	Cotyledon colour	Testa colour	Total saponin content
BG-1017	Granada	5.08	yellow	green	1245
BG-1023	Córdoba	6.16	yellow	beige	1269
BG-1048	Tenerife	2.40	yellow	beige	1045
BG-1055	Jaen	6.16	yellow	beige	1044
BG-1078	Palencia	5.48	yellow	beige	1080
BG-1119	Guadalajara	6.64	yellow	beige	1100
BG-1150	Zamora	6.76	yellow	green	1049
BG-1409	Albacete	6.92	yellow	beige	1269
BG-1415	Baleares	4.72	yellow	beige	1175
BG-1431	Valladolid	2.28	yellow	brown	654
BG-1482	Cuenca	6.52	yellow	green	910
BG-1805	Madrid	5.08	yellow	beige	1163
BG-4245	León	3.20	yellow	brown	771
BG-4249	Soria	5.60	yellow	beige	854
BG-4250	Burgos	2.36	yellow	green	1221
BG-8982	Salamanca	5.72	yellow	beige	1102
BG-11086	Guadalajara	3.56	yellow	beige	1028
BG-11095	Ciudad Real	4.45	yellow	beige	937
BG-16363	Las Palmas	1.28	red	brown	750
BG-16366	Las Palmas	2.64	red	brown	1023

TABLE 3.2. Effect of seed size on the total saponin content (mg kg^{-1}) of *Lens culinaris* Medik.

Seed size	Number of cultivars	Mean	Range
macrosperma	12	1105[a]	854 - 1269
microsperma	8	929[b]	654 - 1221

Macrosperma, 100 g seed > 4.5 g. *Microsperma*, 100 g seed < 4.5 g.
Different superscripts in a column indicate a significant difference ($P < 0.05$).

TABLE 3.3. Effect of seed testa colour on the total saponin content (mg kg^{-1}) of *Lens culinaris* Medik.

Seed testa colour	Number of cultivars	Mean	Range
beige	12	1106[a]	854 - 1269
green	4	1089[a]	910 - 1245
brown	4	800[b]	654 - 1023

Different superscripts in a column indicate a significant difference ($P < 0.05$)

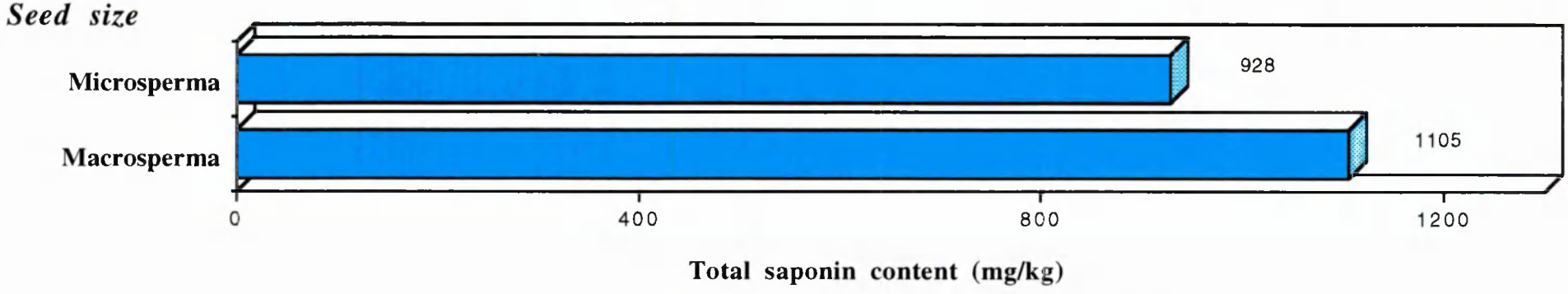


FIGURE 3.2. Effect of seed size on the total saponin content (mg/kg) of lentils.

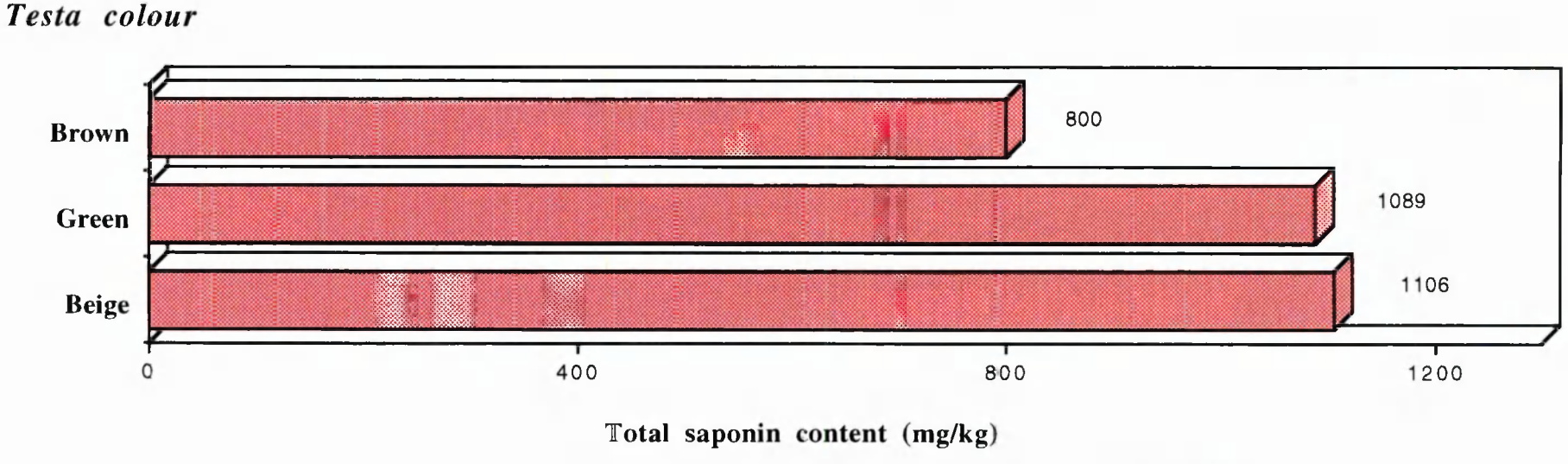


FIGURE 3.3. Effect of seed testa colour on the total saponin content (mg/kg) of lentils.

3.5 DISCUSSION

It has been shown that lentil is a legume with a relatively low saponin content (range: 654 - 1269 mg kg^{-1}) when compared to soyabean, haricot or kidney bean, which have saponin levels of 6500, 4100 and 3500 mg kg^{-1}, respectively. Nevertheless, lentil has a saponin level comparable to that of pea (1100 mg kg^{-1})[50] and high when compared to lupin, whose saponin content ranged from 379 to 740 mg kg^{-1} (see section 2.4.2).

The results reported here are in good agreement with those reported by Kim and Okubo[265], who observed that the content of group B saponins, which have soyasapogenol B as aglycone[45], in soyabeans showed significant differences depending on seed size. Also according to these authors, soyabeans with yellow coats showed a higher saponin content than mixed and brown-coated seeds.

Tsukamoto *et al.*[49] studied the genetic and chemical polymorphisms of saponins in soyabean seed. The chemical and genetic data showed that the directed manipulation of soyabean saponin composition is a possibility for the future.

The work described here provides for the first time quantitative results on the effect of seed size and testa colour on saponin content of *Lens culinaris* Medik. This type of information should be useful in the screening and selection of low or high saponin-containing varieties of lentil as part of any future breeding programme for the manipulation of levels of these biologically active compounds.

3.6 CONCLUSIONS

- The total saponin contents in 20 samples of *Lens culinaris* Medik., which were grown over 5 years in 18 provinces of Spain, ranged from 654 to 1269 mg kg^{-1}.

- Both soyasaponin I and soyasaponin VI were detected in the 20 cultivars of Spanish lentils.

- The changes observed in the saponin content of the Spanish lentils were found to depend on both seed size and testa colour.

- *Macrosperma* lentils were found to have a significantly higher saponin content than *microsperma* seeds.

- The total saponin content of lentil seeds with a brown testa was significantly lower than those with beige or green testa.

IV Isolation of a Novel Saponin

4.1 INTRODUCTION

The isolation of pure saponins from plant materials has proved difficult. Saponins are generally isolated by extraction with organic solvents. The dried plant material is first extracted with acetone, hexane or chloroform, preferably using a Soxhlet extractor, to remove lipids, pigments, etc. The residue is then further extracted with methanol which removes saponins, along with many other compounds such as simple sugars, oligosaccharides, and flavonoids. Various schemes have been devised for isolating the saponins. For example, Domon and Hostettman[271] have described the preferential extraction of bisdesmosidic saponins with methanol. According to these authors, the monodesmosides are extracted most efficiently with water. Kitagawa *et al.*[97,272] noted the almost complete extraction of the soyasaponins bisdemosides A_1 and A_2 into the water layer of a butan-1-ol-water partition of a crude soya extract, whereas the monodesmosides (soyasaponins I, II and III) were found in the butan-1-ol fraction.

One of the most recent advances in the large-scale isolation of saponins from plant tissue has involved 'flash chromatography', a hybrid of preparative high pressure liquid chromatography and gravity-fed column chromatography (see section 1.2.4.1). Price and Fenwick[95], for instance, isolated soyasaponin I from pea (*Pisum sativum*) using this technique. They applied their methanol extract (redissolved in water) to a 'flash chromatography' column (glass column) containing a reversed-phase octadecylsilane bonded to silica gel. The column was washed with water and then eluted with methanol. The crude saponin fraction was then purified by using normal phase silica gel and elution with chloroform: methanol (1:1 by volume).

The importance of reversed-phase 'flash chromatography' in this context can be attributed to the use of polar solvents and hence to the high solubility of the crude saponin mixture; moreover, the accessibility of the column packing allows easy replacement and/or repacking.

However, in general, no single technique is sufficient to yield pure saponins, and a sequential application of several techniques is necessary. Nevertheless, the integration of 'flash chromatography' and normal phase silica gel chromatography is the preferred procedure in most cases.

Taking into account all the considerations mentioned above, the aim of this work was to isolate a novel saponin from *Lupinus angustifolius* flour in sufficient quantity to enable its structure to be elucidated. From mass spectra of the crude saponin extract, the new saponin appears to have soyasapogenol B as aglycone and a relative molecular mass (M_r) of 1104 (see section 2.4.1).

4.2 MATERIALS

Lupins (*Lupinus angustifolius*, cultivar Warrah) were obtained from a crop grown in Kapinnie (South Australia) during 1992.

Standard samples of saponins and chemicals were the same as those described in Section 2.2.

D_5-pyridine (99.5% *D*) + 0.1% tetramethylsilane was purchased from Goss Scientific Instruments, Great Baddow, Essex, UK.

Flash chromatography packings were supplied by J. T. Baker Chemical Co., Philipsburg, New Jersey, USA. Both normal phase silica gel 60 packing and normal phase thin layer chromatography plates were purchased from E. Merck, Darmstadt, Germany. Reversed-phase thin layer chromatography plates were provided by Whatman International Ltd., Maidstone, UK. Techelut SPE columns C_{18} (ODS) were supplied by Hplc Technology, Macclesfield, UK.

4.3 METHODS

4.3.1 Isolation and purification

The whole process of isolation and purification of the novel saponin present in *Lupinus angustifolius* seed is summarized in Figure 4.2 at the end of section 4.3.1

Ground lupin flour (500 g) was mixed with sand (1000 g) and Soxhlet extracted with chloroform (5 l) for 16 h. The air-dried mixture was then Soxhlet-extracted with methanol (7 l) for 30 h.

The resultant methanol extract was monitored by thin layer chromatography (TLC) using reversed-phase plates (Whatman KC_{18}; 20 x 20 cm; layer thickness: 0.25 mm) developed in methanol:water (3:2) to a distance of 12 cm and air dried. The plates were then visualised by spraying with *p*-anisaldehyde/glacial acetic acid/ 97% w/v sulphuric acid (1:100:2) and gentle heating with hot air for 5 min. The presence of soyasaponins I and VI in the methanol solution was confirmed with the use of standard samples.

Five µl of the methanol extract were added to a drop of glycerol on the fast atom bombardment (FAB) copper probe tip. Mass spectra were obtained using a Kratos MS 9/50TC mass spectrometer by bombardment of the sample with an ion beam of xenon produced by an Ion-Tech 11 NF atom gun operating at 9 kV (nominal). Since saponin ions are more stable in the negative-ion mode, only the negative ion spectrum was recorded using a UV galvanometer recorder. FAB mass spectra of the methanol solution revealed the presence of soyasaponin I (M_r: 942), soyasaponin VI (M_r: 1068) and the novel saponin (M_r: 1104).

The methanol extract was evaporated to dryness *in vacuo*, dissolved in water (500 ml) and applied to a reversed-phase flash chromatography column (100 g; octadecylsilane bonded to silica gel; J.T. Baker C_{18}), which had previously been conditioned first with methanol (500 ml) and then with water (500 ml). The column was eluted sequentially

with water (500 ml) and methanol (500 ml) under a pressure of 15 psi (1 psi ≈ 6.9 kPa).

Fractions (20 ml) of both the water and methanol eluates were monitored by TLC using the conditions mentioned above. Both soyasaponins I and VI were found principally in the methanol eluate.

FAB mass spectra of these water and methanol eluates showed the new saponin to be mainly present in the former eluate whereas the latter was rich in soyasaponin I and soyasaponin VI. The water fractions were combined and freeze-dried.

Five grams of the freeze-dried material was mixed with sand (10 g), dissolved in methanol and evaporated to dryness by a stream of N_2. The dry mixture was applied to the top of a glass column packed with normal phase silica gel (100 g; Merck silica gel 60; particle size: 0.063 - 0.200 mm; 70-230 mesh ASTM) in chloroform:methanol:water (65:35:10, lower phase) over a layer of sand (5 g). A piece of filter paper was placed above the mixture of sample and sand and, finally, a separating funnel containing the eluting system (chloroform:methanol:water; 65:35:10, lower phase) was added to the top of the column and the sample eluted. A fraction collector (Frac-300; Pharmacia Fine Chemicals) was connected to the end of the column and fractions of approximately 10 ml each were collected.

The fractions (grouped in sets A to L) were monitored by TLC using normal phase silica gel plates (Merck silica gel 60; 20 x 20 cm; layer thickness: 0.20 mm) developed in chloroform:methanol:water (65:35:10, lower phase) to a distance of 12 cm. The plates were then air dried and visualised by spraying with *p*-anisaldehyde/glacial acetic acid/ 97% w/v sulphuric acid (1:100:2) and gentle heating with hot air for 5 min. Individual spots showed purple or blue colours, which are characteristic of steroids and triterpenoids (Figure 4.1).

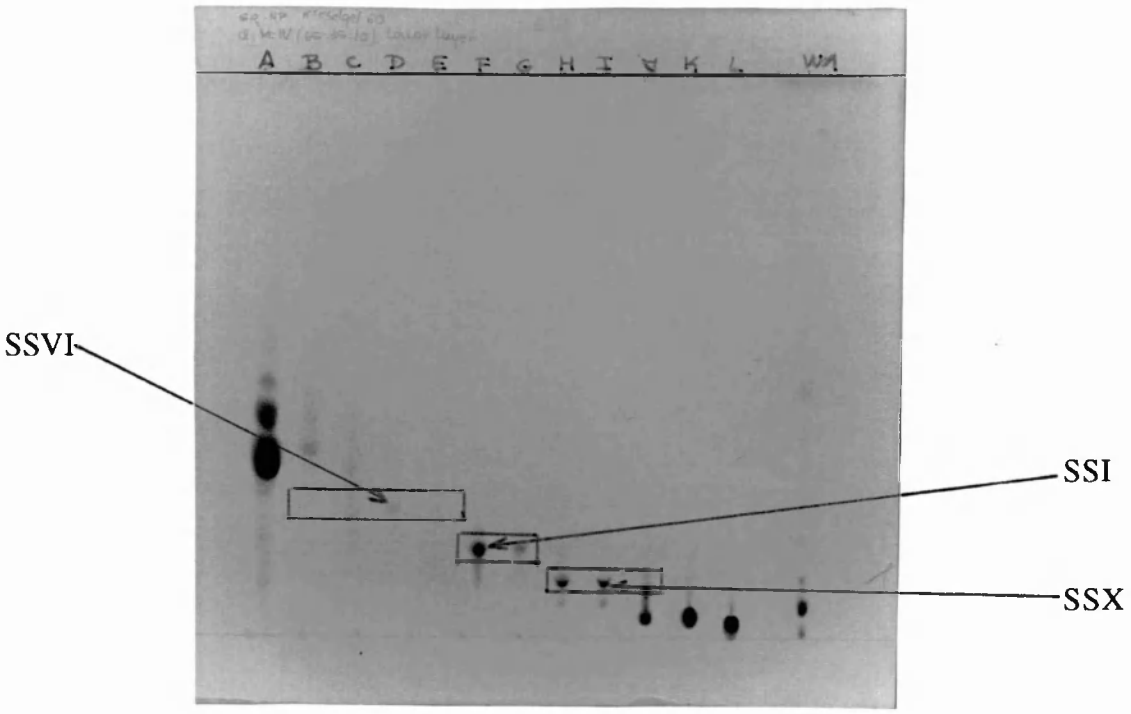


FIGURE 4.1. Thin layer chromatogram (silica gel 60 plate; solvent system, chloroform: methanol:water, 65:35:10, lower phase) of all the sets of fractions (A to L) from the elution with chloroform:methanol:water (65:35:10, lower phase) of the freeze-dried material containing the novel saponin applied to a normal phase silica column. W1 corresponds to the original freeze-dried sample dissolved in the same mixture of chloroform:methanol:water mentioned above.

SSVI: soyasaponin VI; SSI: soyasaponin I; SSX: new saponin

FAB mass spectra of the different sets of fractions and TLC, using the conditions described above and standard samples, showed soyasaponin VI to be present in sets B, C, D and E, whereas soyasaponin I was found in sets F and G (Figure 4.1). FAB mass spectra of sets H, I and J revealed the presence of the new saponin (Figure 4.1). For

simplicity, only sets of fractions containing principally the new saponin (H and I) free from large contamination by other compounds, such as sugars, were combined, concentrated to dryness *in vacuo* and dissolved in methanol (10 ml).

The methanol solution obtained above was evaporated to dryness *in vacuo*, redissolved in water and freeze-dried. The powdered sample (100 mg) was dissolved in 10% aqueous methanol and eluted through a flash column chromatography packed with reversed-phase silica (10 g) from disposable Techelut SPE columns C_{18} (ODS) which had previously conditioned first with methanol (10 ml) and then with water (10 ml). The elution was carried out according to the following gradient: 100% water, 0% methanol (10 ml); 90% water, 10% methanol (10 ml); 80% water, 20% methanol (10 ml); 70% water, 30% methanol (10 ml); 60% water, 40% methanol (10 ml); 50% water, 50% methanol (30 ml), 0% water, 100% methanol (30 ml) under a pressure of 5 psi.

Fractions (0.5 ml) were collected and monitored by TLC using reversed-phase KC_{18} plates developed in methanol/water (3:2). Those fractions containing only the new saponin were combined, evaporated to dryness in a vial by a stream of nitrogen, dissolved in water and freeze-dried.

Several repetitions of the whole process (Figure 4.2) yielded ~ 25 mg of the new saponin. The sample was then divided in several portions to carry out both normal and reversed-phase TLC, FAB-MS, electrospray mass spectrometry, acid hydrolysis and GC-MS of sapogenol and nuclear magnetic resonance spectrometry in order to elucidate the structure of the new saponin.

FIGURE 4.2 Isolation and purification of a novel saponin from *L. angustifolius* seed.

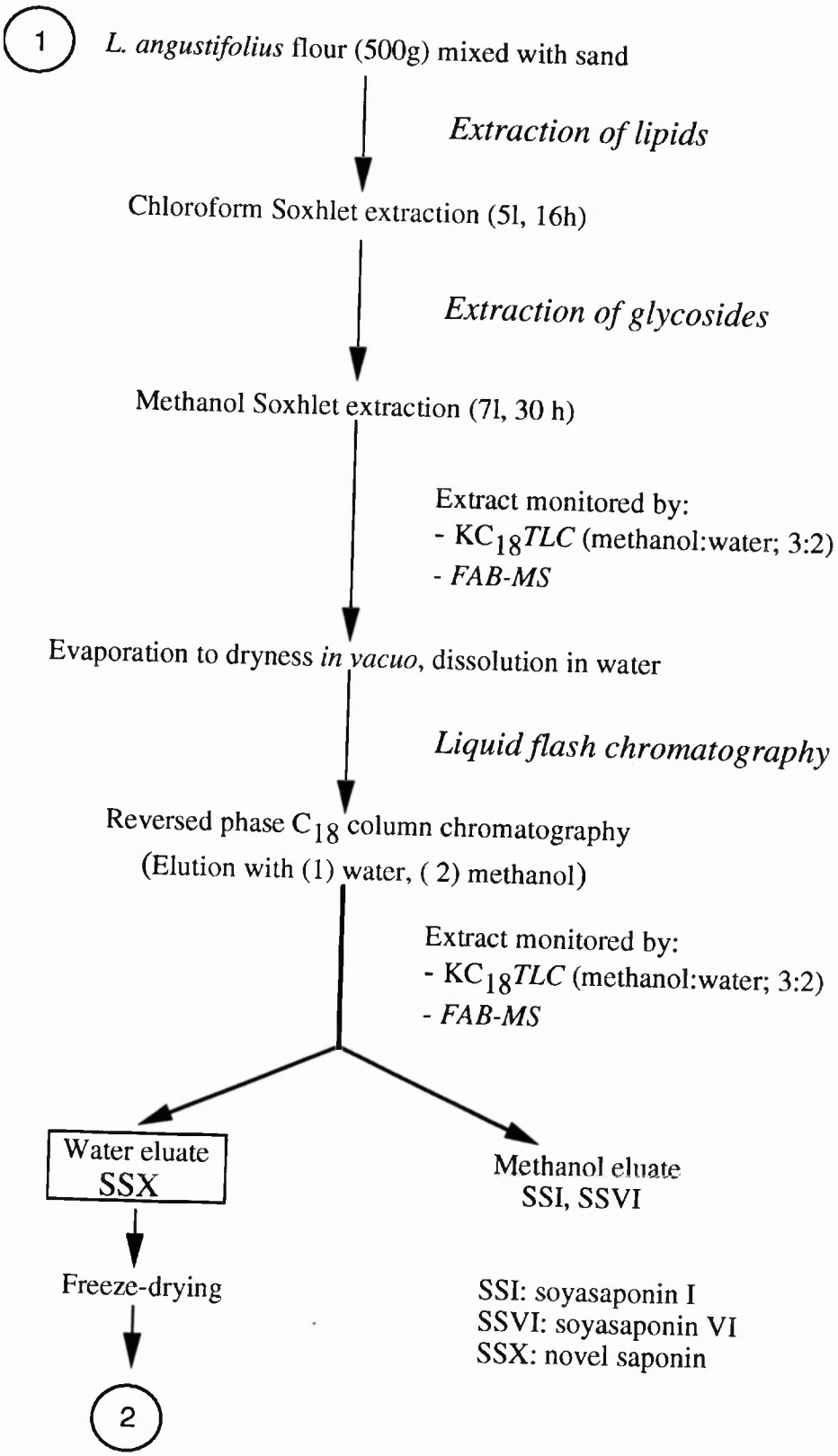


FIGURE 4.2 Isolation and purification of a novel saponin from *L. angustifolius* seed (continued).

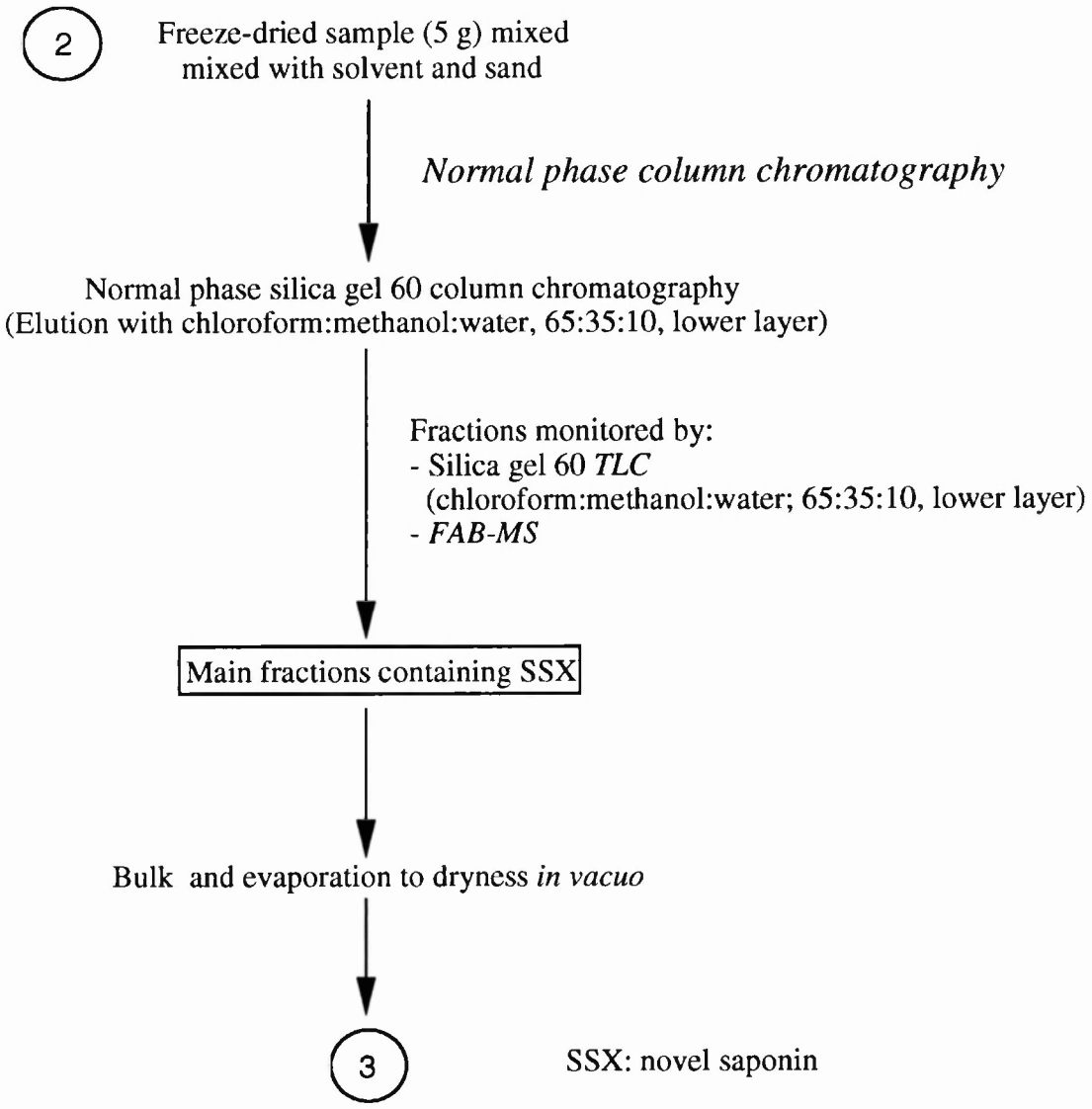
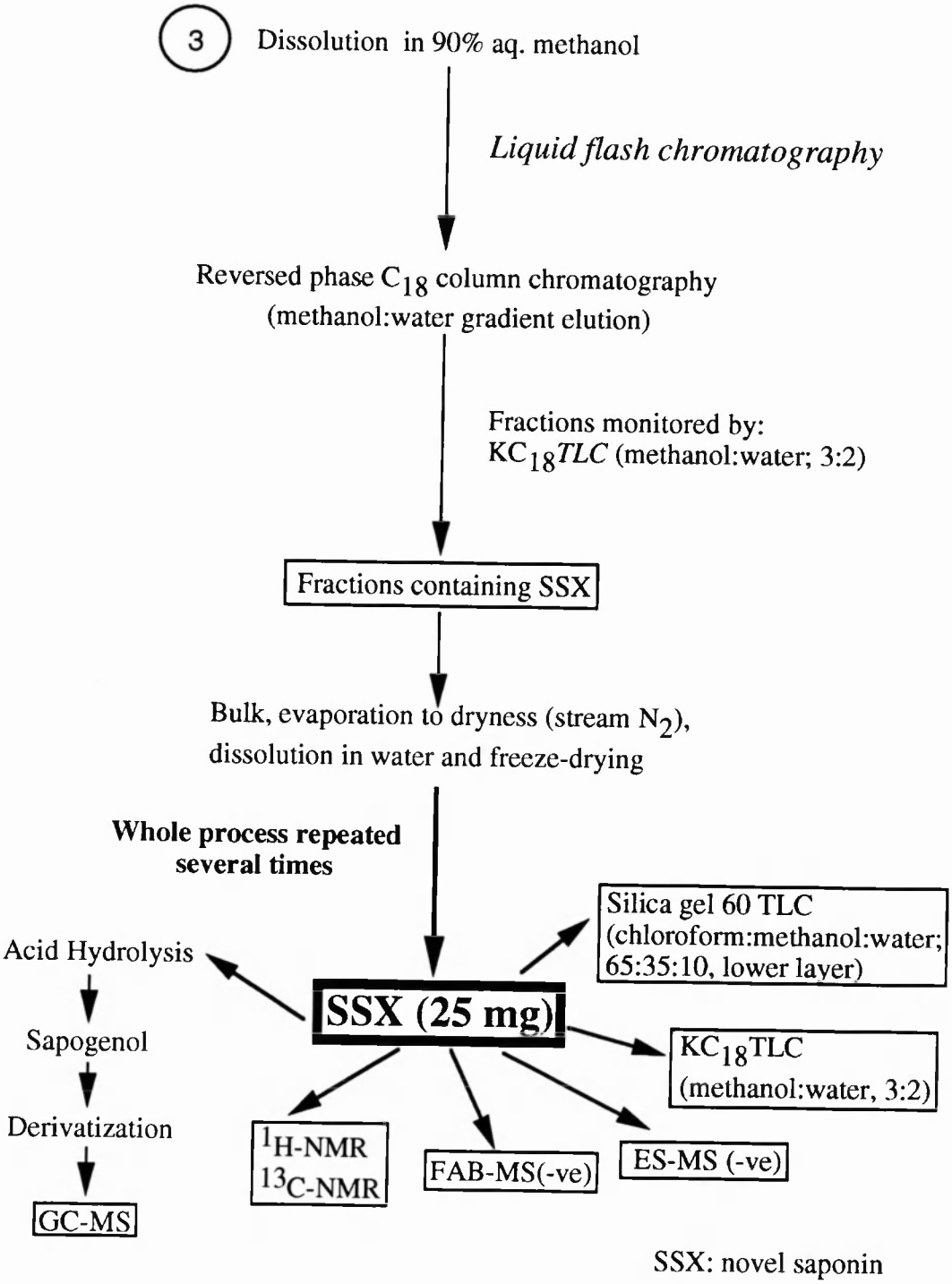


FIGURE 4.2 Isolation and purification of a novel saponin from *L. angustifolius* seed (continued).



4.3.2 TLC

Solutions of the new isolated saponin in aqueous methanol (1 mg ml^{-1}) and in chloroform: methanol:water mixtures (1 mg ml^{-1}) were monitored by reversed- and normal phase TLC using the conditions mentioned above.

4.3.3 FAB-MS

Five µl of a solution of the new saponin in methanol (1 mg ml^{-1}) was added to a drop of glycerol on the fast atom bombardment (FAB) copper probe tip. A FAB mass spectrum in the negative-ion mode was obtained using a Kratos MS 9/50TC mass spectrometer by bombardment of the sample with an ion beam of xenon produced by an Ion-Tech 11 NF atom gun operating at 9 kV (nominal).

4.3.4 Electrospray mass spectrometry (ES-MS)

An ES mass spectrum in the negative-ion mode of a solution of the new saponin in acetonitrile:water (1:1) (40 µg/ml) was obtained on 20 µl loop injections directly into the ES source (Fisons Instruments VG Platform).

4.3.5 Acid hydrolysis of saponin and GC-MS of sapogenol

The new saponin (1 mg) was dissolved in methanol (1 ml) and acid hydrolysed to produce a sapogenol (see section 2.3.4), which was derivatized as described in section 2.3.5. Peak identity of the derivatised sapogenol was determined by GC-MS (see section

2.3.6).

4.3.6 Nuclear magnetic resonance spectrometry

Proton and ^{13}C-NMR spectra were obtained on a Jesl JNM-EX400 spectrometer, operating at 399.65 and 100.4 MHz, respectively. The sample (~ 20 mg) was dissolved in D_5-pyridine (99.5% *D*) + 0.1% trimethylsilane.

4.4 RESULTS AND DISCUSSION

4.4.1 TLC

Both normal and reversed-phase TLC of the isolated saponin revealed a single purple spot. R_f values for the new saponin in normal and reversed-phase TLC plates were 0.23 and 0.59, respectively.

4.4.2 FAB-MS

The FAB mass spectrum in the negative-ion mode of this compound (Figure 4.3) exhibited a major molecular ion at m/z 1103, with a loss of one deoxyhexose (ion at m/z 957). Ions with less abundance at m/z 941 and m/z 795 were indicative of losses of one hexose: [M - (H+h)]$^-$ and one deoxyhexose plus one hexose: [M - (H+d+h)]$^-$ respectively (Figure 4.3).

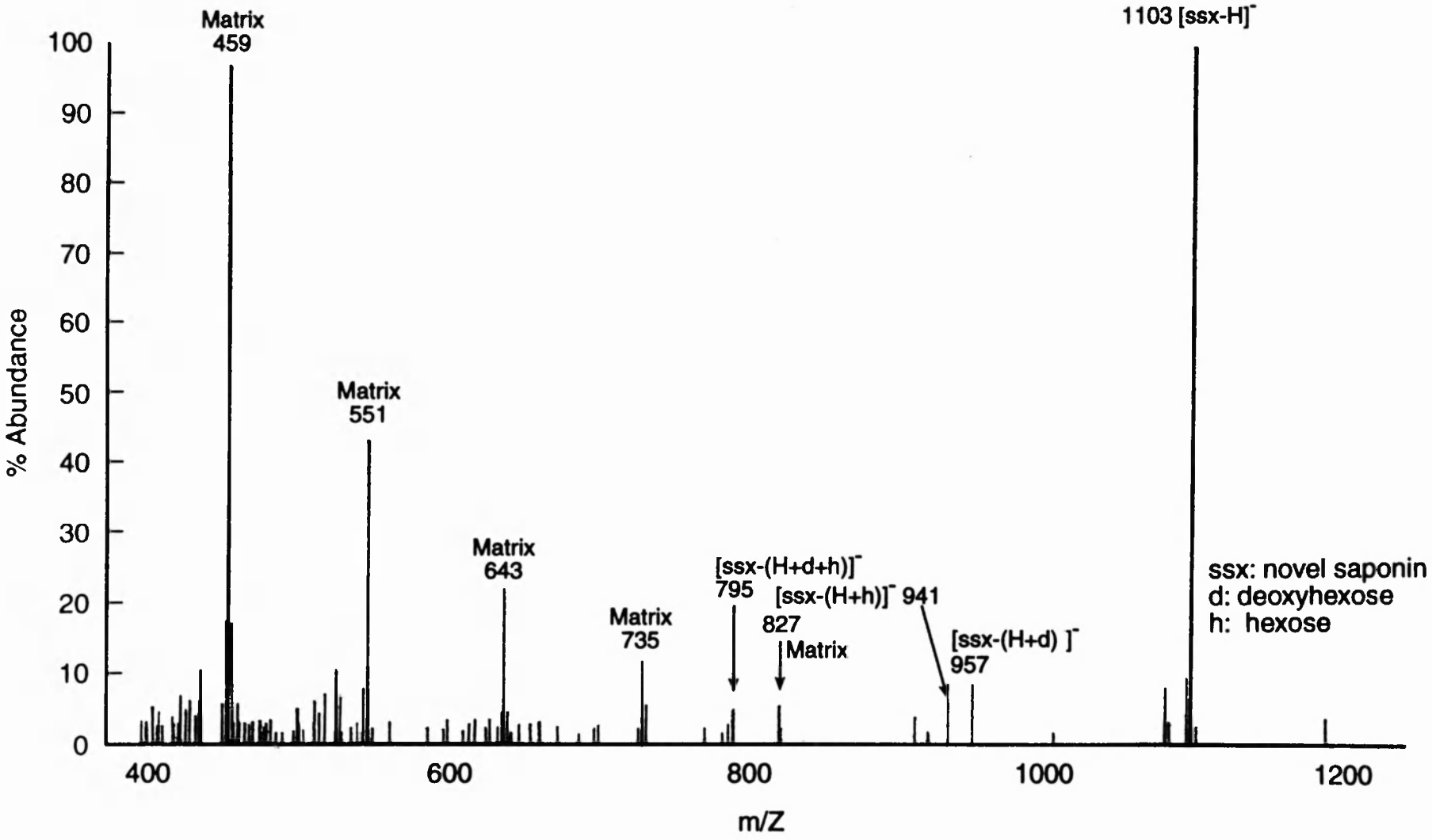


FIGURE 4.3 FAB mass spectrum in the negative mode of the novel saponin.

4.4.3 ES-MS

The new saponin yielded a major ion at m/z 1103.8, as expected (Figure 4.4). Ions with less abundance at m/z 957.5 and m/z 795.3 would confirm the loss of one deoxyhexose and one deoxyhexose plus one hexose, respectively, shown in the previous section.

4.4.4 GC-MS

Analysis of the aglycone using coupled gas chromatography-mass spectrometry confirmed the relative molecular mass as 458 (soyasapogenol B) (Figure 4.5). Large peaks occurred at m/z 306 and 291 (Figure 4.6), which are characteristic of the Retro Diels- Alder (RDA) (Figure 4.5) cleavage of C_{12}-C_{13} unsaturated triterpenes. The former peak (m/z 306) corresponds to the RDA fragment (m/z 234) plus a trimethylsilyl (TMS) group from the derivatisation procedure (see section 2.3.5). The latter (m/z 291) is characteristic of the silylated RDA fragment with a loss of one of the methyl radicals of the TMS group, which is a common fragmentation.

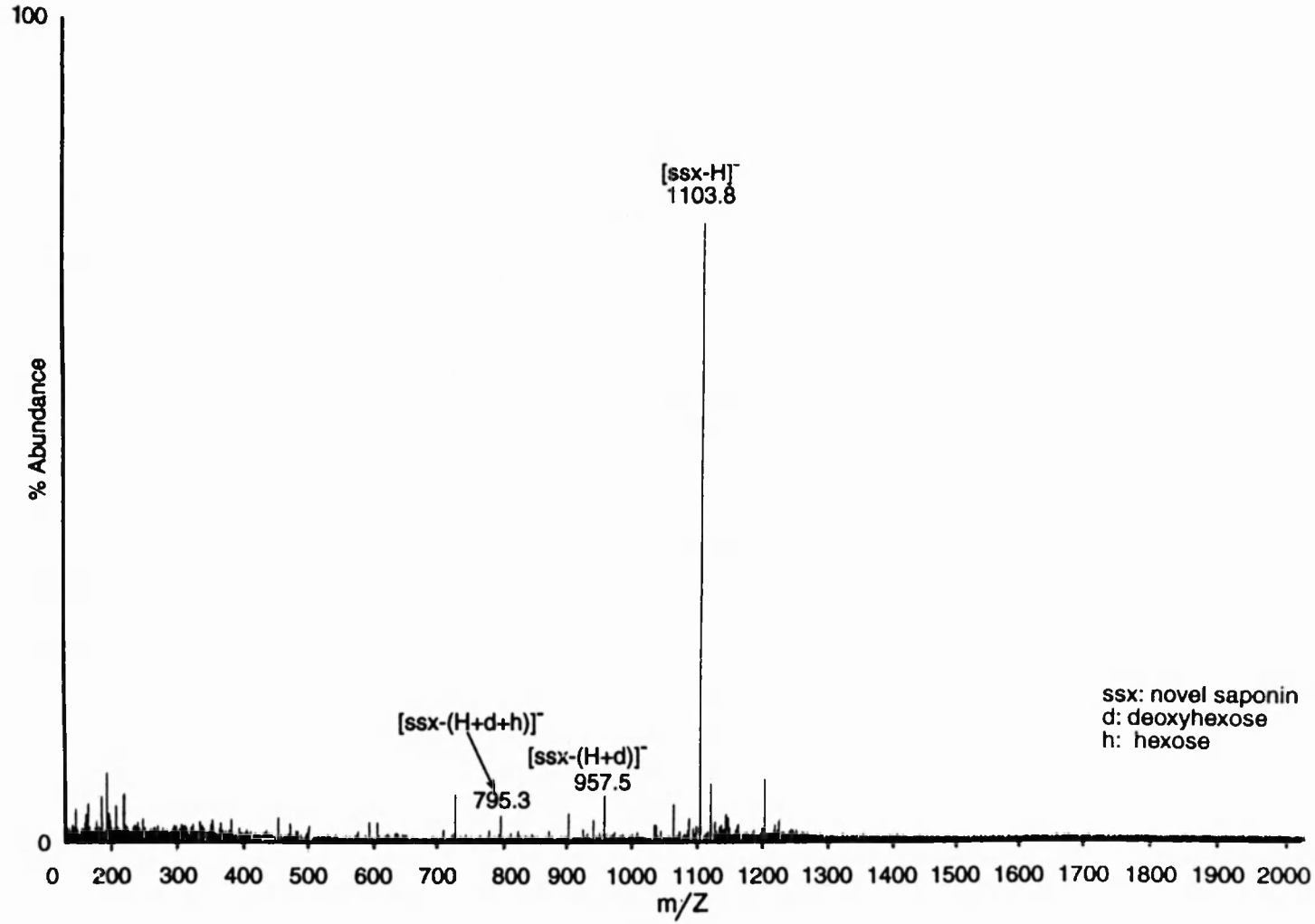


FIGURE 4.4 ES mass spectrum in the negative mode of the novel saponin.

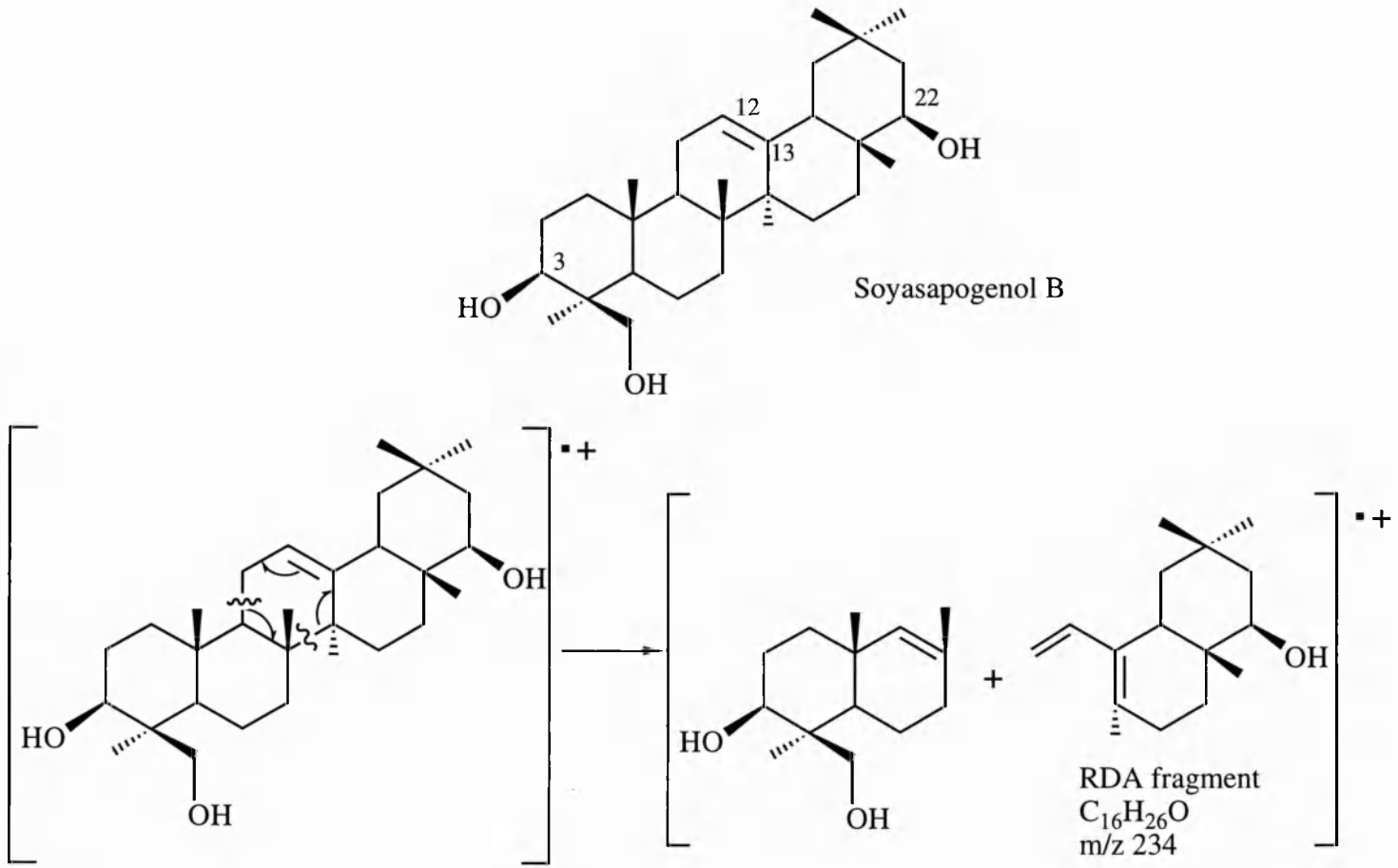


FIGURE 4.5. Mechanism of Retro-Diels Alder (RDA) Fragmentation under electron impact.

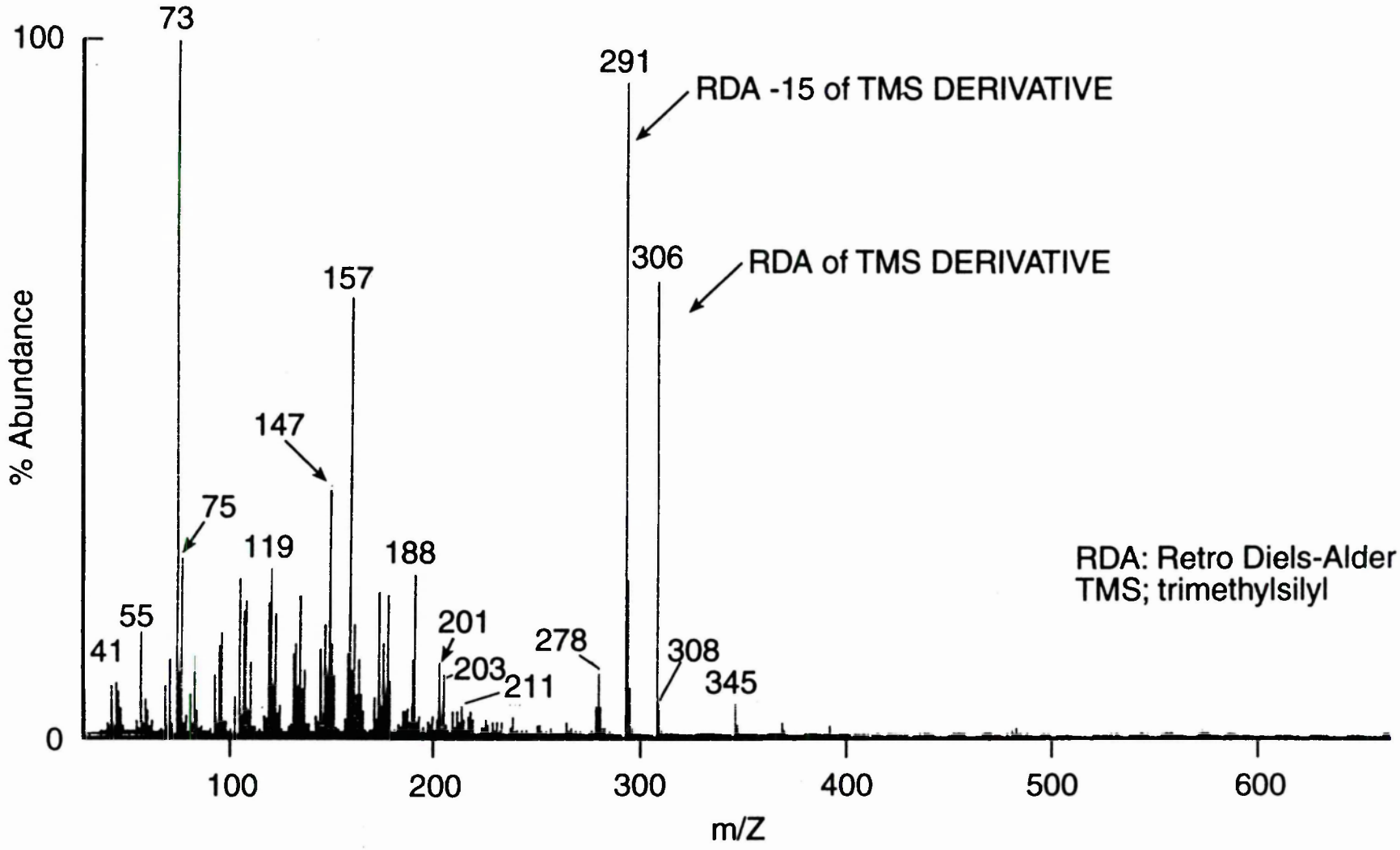


FIGURE 4.6 GC mass spectrum of the sapogenol derived from the novel saponin.

4.4.5 ^{1}H and ^{13}C-NMR

Signals in the ^{1}H and ^{13}C NMR spectra (Figure 4.7) were much weaker than expected from the amount of sample analysed. It was noted that not all the sample would dissolve in the D_5-pyridine solvent but the amount undissolved did not seem large. Although the mass spectral readings indicated the presence of a novel saponin in the sample, the weak signals in the NMR suggest that an unidentified impurity was present in the sample and because this impurity was not detected by the TLC spray reagent used, it can be inferred that it is of inorganic origin, and by its nature would be unlikely to co-elute with the saponin in the two different TLC systems used. If the impurity were to be of organic origin and to also co-elute with the saponin in the TLC systems, then much stronger signals would be expected in the NMR spectrum for the weight of sample analysed.

The presence of this impurity made complete structural elucidation of the novel saponin by NMR impossible. No further work was attempted to purify further the novel saponin.

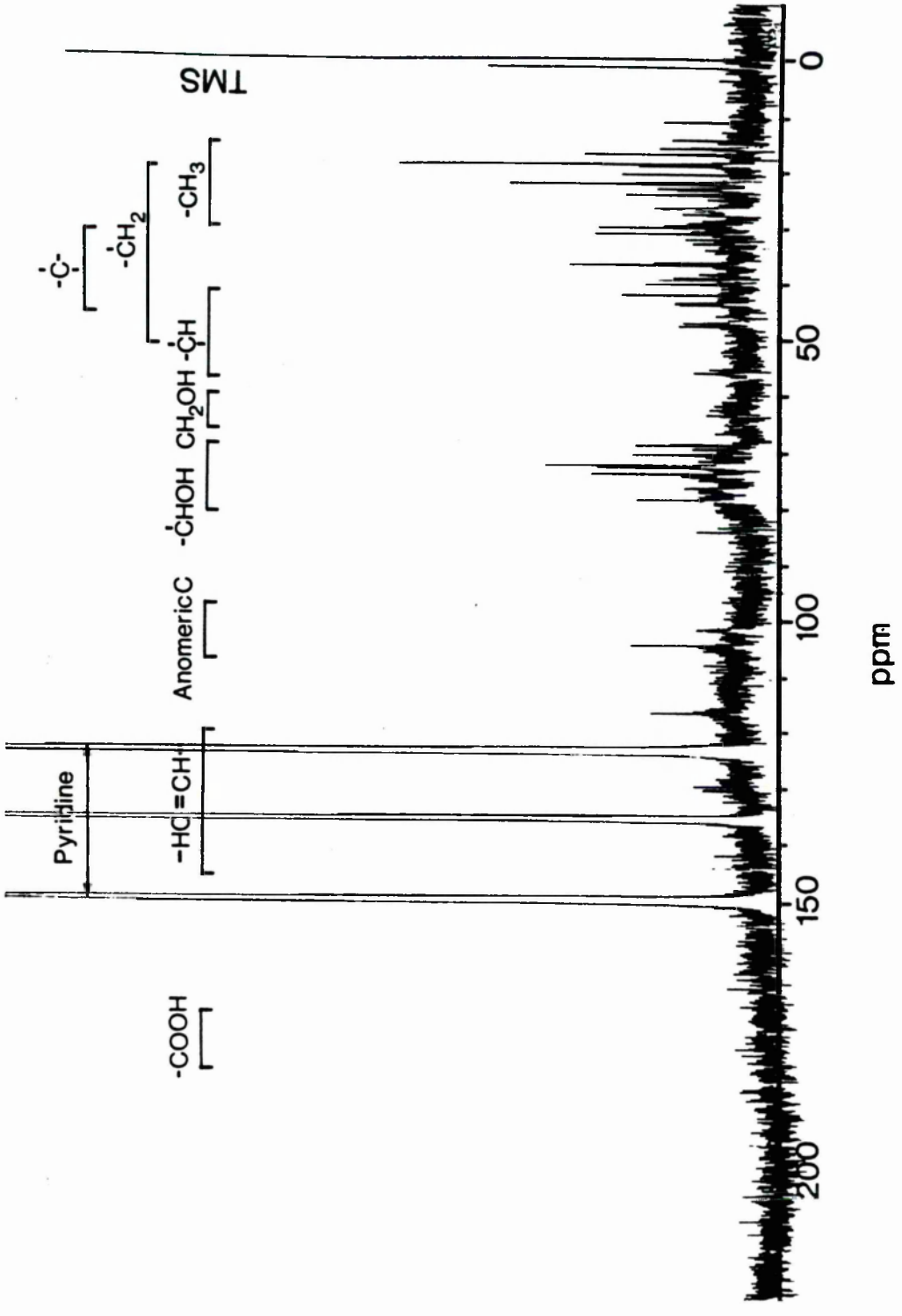


FIGURE 4.7 ^{13}C NMR spectrum of the novel saponin.

4.5 HYPOTHESIS

Apart from a signal from the deprotonated molecule, both the FAB and ES mass spectra in the negative-ion mode of the novel saponin exhibited an ion at m/z 957, which is indicative of the loss of one deoxyhexose. The FAB mass spectrum also displayed a peak at m/z 941, which is indicative of the loss of one hexose. An ion at m/z 795, which involves the loss of one deoxyhexose plus one hexose, was also present (see sections 4.4.2 and 4.4.3). Bearing in mind the composition of the glycosidic moiety of soyasaponin I: deoxyhexose (rham), hexose (gal) and uronic acid (glcUA) (Figure 4.9), and, since the difference in relative molecular masses between the new saponin (1104) and soyasaponin I (942) is equivalent to an hexose (162), it can be postulated that the saponin is a novel *O*-22 hexose conjugate of soyasaponin I (Figure 4.9).

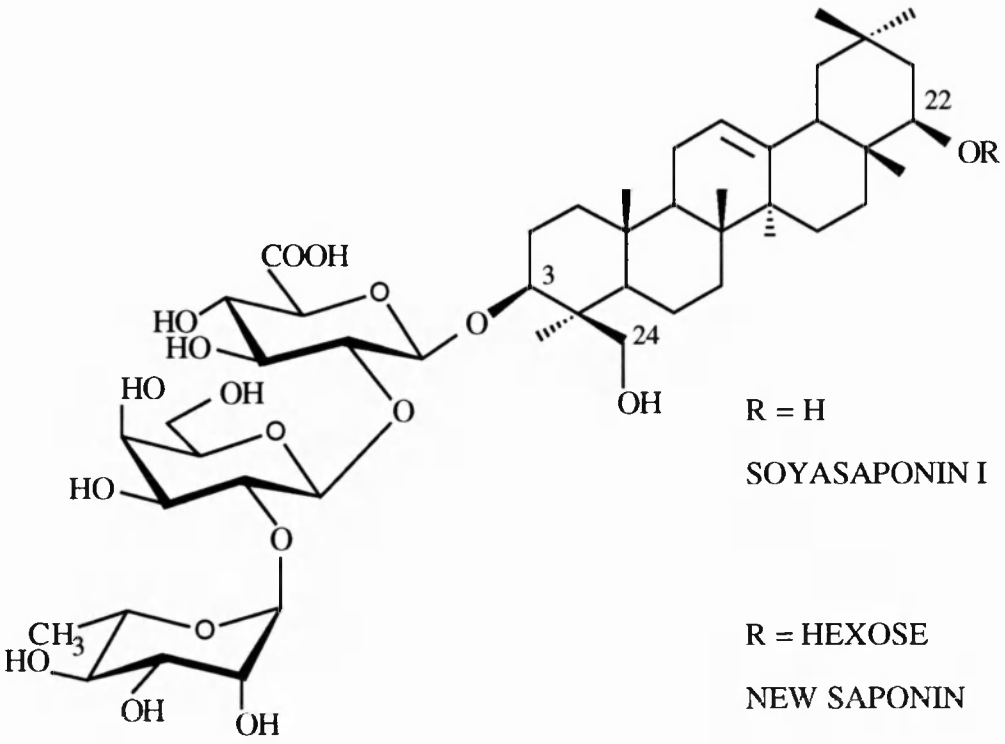


FIGURE 4.9 Chemical structure of soyasaponin I and possible chemical structure of the novel saponin present in *L. angustifolius* seed.

The extra hexose of the new saponin, in relation to soyasaponin I, could have not been linked to the deoxyhexose as a terminal sugar (Figure 4.9) since no explanation could be given for the peak at m/z 957 found in the mass spectra (Figures 4.3 and 4.4). It is unlikely that the additional hexose of the novel saponin could be intercalated between the uronic acid and the other hexose, or between the latter and the terminal deoxyhexose (Figure 4.9) because the peak at m/z 941 from the FAB mass spectrum (Figure 4.3) would not be justified, unless the deoxyhexose, instead of being located at the end of the saccharide chain, was linked to the C-22 of the aglycone (Figure 4.9), which has not been reported in other saponin structures.

The only two other possible positions for the extra hexose of the new saponin would be either linked to the C-24 of the aglycone or branched to the other hexose unit in the saccharide chain (Figure 4.9). However, since no previous soyasaponin has been reported to have a sugar linked to the C-24 oxygen of the aglycone or a branched glycosidic moiety, the novel saponin is strongly believed to be a new bisdesmoside of soyasapogenol B (Figure 4.9). Yoshikawa *et al.*[273] have reported a bisdemoside of soyasapogenol B, sophoraflavoside I, from *Sophora flavescens*, an Eastern medical herb and this compound was characterized as 3-*O*-[α-L-rhamnopyranosyl(1-2)-ß-D-galacto-pyranosyl(1-2)-ß-D-glucuronopyranosyl]-22-*O*-[ß-D-glucopyranosyl(1-2)-α-L-arabinopyranosyl] soyasapogenol B, so there is a precedence for an hexose linked to the 22-C oxygen of soyasapogenol B.

4.6 CONCLUSIONS

- A new saponin has been isolated from Australian *L. angustifolius* using a combination of reversed-phase silica (C_{18}) with aqueous methanol and normal phase

silica with chloroform:methanol:water mixtures.

- Both FAB and ES mass spectra in the negative-ion mode exhibit a quasi-molecular ion at m/z 1103. Therefore the relative molecular mass of the novel saponin was deduced to be 1104.

- Soyasapogenol B was confirmed to be the aglycone of the new saponin by GC-MS.

- Both reversed- and normal phase TLC plates displayed distinct single spots indicating a high degree of organic purity.

- Since very weak NMR spectra were obtained, a complete structural elucidation of the new saponin has not been possible. However, from its mass spectra, it is postulated to be a new *O*-22 hexose conjugate of soyasaponin I.

V Determination of Saponins in Lupin Seed Using High-Performance Liquid Chromatography

5.1 INTRODUCTION

The quantitative determination of saponins has for a long time posed problems to the analytical chemist[35]. A number of chromatographic methods have been used for saponin analysis and especial attention has focused on the use of gas chromatography but it has the limitation that it can only be used for the separation and quantification of the aglycone portion of the saponin after hydrolysis and suitable derivatization (see Section 2.3), which involves both the loss of structural information about the glycosidic portion of the molecule and potential loss of material during hydrolysis and derivatization.

Since relatively little is known about the effect of both industrial and domestic processing on the fate of the saponins in food and, with recent work demonstrating a relationship between chemical structure and biological activity[185-187,259], there is a requirement for analytical methods that can measure the individual saponins as they exist in the food matrix. However, the development of techniques for the analysis of these intact saponins, such as high-performance liquid chromatography (HPLC), has been limited due to (a) the difficulties with the detection of triterpene saponins, which do not contain a UV-chromophore, (b) the lack of appropriate standards and (c) the relative instability of some saponins. There have been several attempts to overcome the detection problems such as the monitoring of underivatized saponins with a light-scattering detector[138]. This mode of detection, however, has some limitations as to the solvents and gradients that can be used[274].

One solution is to derivatize the saponins with a chromophore which facilitates UV detection. The method of Slacanin *et al.*[274] involves the derivatization of saponins with 4-bromophenacyl bromide which can be completed with any saponin containing a free carboxylic acid group in the aglycone or sugar moiety (Figure 5.1).

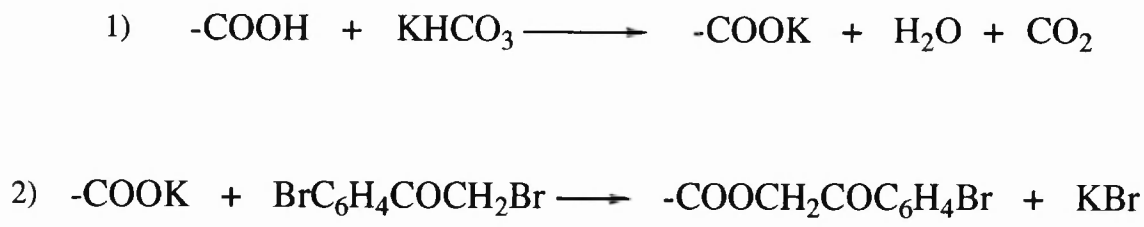


FIGURE 5.1. Chemical reactions for the derivatization of saponins involved in the HPLC method developed by Slacanin *et al.*[274].

Using the method developed by Slacanin *et al.*[274] with some modifications, Oleszek *et al.*[141] successfully detected the derivatized saponins from alfalfa roots, which contain both one and two carboxylic acid functions, after a single chromatographic run on a silica C_{18} column. These authors postulated that the method could be applied for the quantification of individual glycosides.

Both soyasaponin I and soyasaponin VI (Figure 5.2), which have been shown to be the main saponins present in *L. angustifolius* (see Section 2.4.1), have a glucuronic acid unit in their glycoside chain. Therefore, these two saponins were theoretically capable of undergoing derivatization with 4-bromophenacyl bromide to be analysed by HPLC according to Oleszek *et al.*[141].

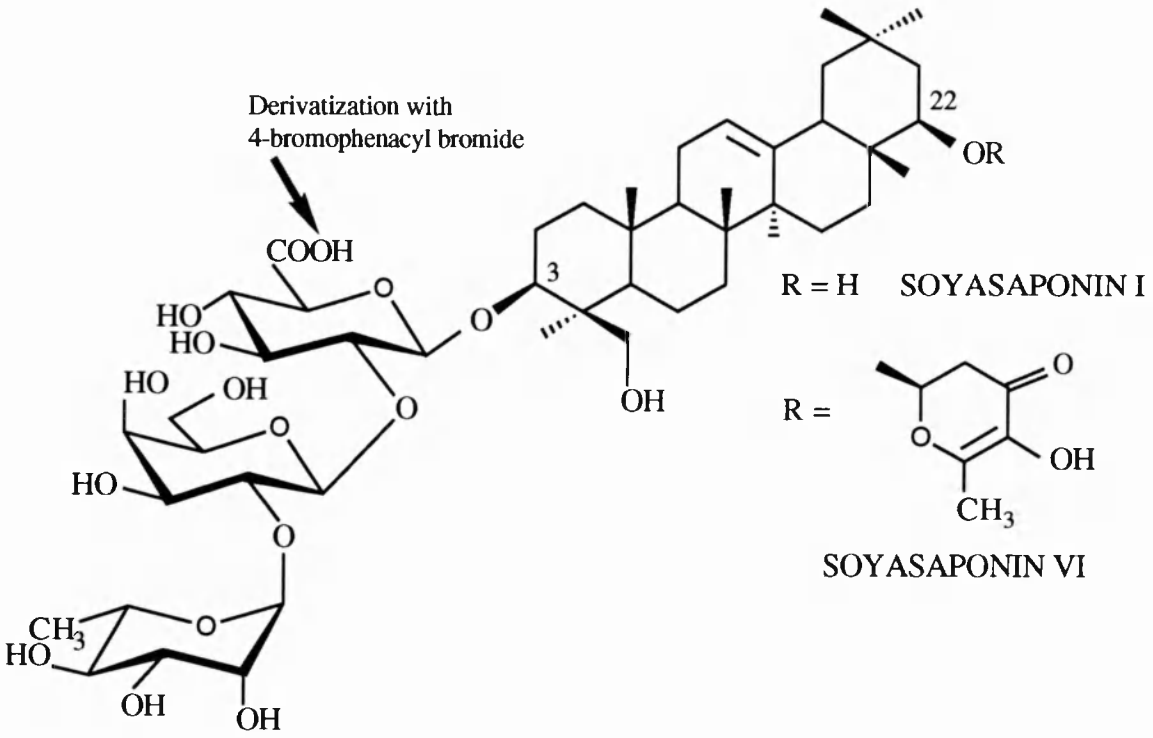


FIGURE 5.2. Chemical structures of soyasaponin I and soyasaponin VI.

On the other hand, Kudou *et al.*[256], Yoshiki *et al.*[257,259] and Okubo *et al.*[258] have separated, on a qualitative basis using HPLC, underivatized 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) saponins from soyabean, runner bean, hyacinth bean and American groundnut. These authors postulated that DDMP saponins were the genuine saponins in all the legume seed mentioned above (see Section 1.2.10.1). DDMP saponins were extracted with 70% aqueous ethanol containing 0.01% EDTA at room temperature. HPLC separation of DDMP saponins was performed on a reversed-phase silica column using an EDTA-acetonitrile-water-acetic acid mixture as the mobile phase and detection was by UV absorption at 292 nm, which is the maximum absorption for these compounds containing a conjugated enone functionality (see Section 1.2.10.2).

Soyasaponin VI is the DDMP conjugate of soyasaponin I (Figure 5.2). As mentioned above, both saponins were detected in *L. angustifolius* seed after hot solvent

extraction with methanol (see Section 2.3.1). According to Okubo and coworkers, soyasaponin VI would be the only saponin present in the lupin seed whilst soyasaponin I would constitute an artifact derived from degradation of its DDMP conjugate during the extraction process. However, the mild extraction process these authors proposed was only studied on a qualitative basis and needs to be carefully tested in order to prove the above-mentioned assumption.

The aims of this work were the quantification of individual saponins from lupin seed (*Lupinus angustifolius*) by HPLC and the comparison of the results obtained with those using the conventional GC method for the analysis of saponins (see Section 2.3). Two different approaches were attempted in order to achieve this purpose, one based on the derivatisation procedure by Oleszek *et al.*[141] and the other on the method using underivatised saponins by Okubo and coworkers[256-259].

5.2 MATERIALS

Lupins (*Lupinus angustifolius*, cultivar Warrah) were obtained from a crop grown in Kapinnie (South Australia) during 1992.

Standard samples of soyasaponins I and VI were the same as those described in Section 2.2. Soyasapogenol B and α-hederin were purchased from Apin Chemicals Ltd., Abingdon, Oxon, U.K. Glucuronic acid was supplied by Sigma Chemical Co., Poole, Dorset, U.K.

All solvents used during the extraction process were of analytical-reagent grade. Methanol, chloroform, acetonitrile, ethanol, butan-1-ol, acetic acid and potassium hydrogen carbonate were purchased from BDH Laboratory Supplies, Poole, U.K. Dichloromethane and ethylenediaminetetraacetic acid (EDTA) were supplied by Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, U.K. Both 4-bromo-phenacyl bromide and 18-crown-6 (1,4,7,10,13,16-hexaoxacyclo-octadecane) were

supplied by Sigma Chemical Co., Poole, Dorset, U.K. Water was purified using a Milli-Q system, Waters Chromatography Division, Millipore Corporation, Milford, U.S.A.

Silica gel Sep-pak cartridges and Techelut SPE C_{18} columns were purchased from Waters Assoc., Milford, U.S.A. and HPLC Technology, Macclesfield, U.K., respectively.

5.3 METHODS

5.3.1 High-performance liquid chromatography of derivatized saponins in *L. angustifolius* seed

The first attempt to analyse saponins from lupin (*L. angustifolius*) seed by HPLC was based on the method developed by Oleszek *et al.*[141] for the qualitative determination of saponins present in alfalfa roots, with some modifications.

5.3.1.1 *Preparation of saponin derivatives*

5.3.1.1.a Saponin standards

Saponin (4 mg) was dissolved in 0.25 ml of methanol, and 2 ml of water containing 1 mg of potassium hydrogen carbonate was added dropwise with occasional sonication. The samples were lyophilized, treated with 1 ml of a mixture of 4-bromophenacyl bromide (3.5 g) and 18-crown-6 (680 mg) in acetonitrile (100 ml), refluxed at 100°C for 90 min and purified on a Silica gel Sep-pak cartridge by eluting with dichloromethane (10 ml) followed by chloroform:methanol (1:1) (10 ml). After evaporation of the latter solvent, the sample was redissolved in 2 ml of methanol and used for HPLC analysis.

5.3.1.1.b Saponin extract from *L. angustifolius* seed

L. angustifolius seed (5 g) was defatted with chloroform and saponins were extracted with methanol as described in Section 2.3.1. The methanol extract was evaporated to dryness *in vacuo* and redissolved in water (5 ml). The aqueous solution was eluted through a Tech Elut SPE C_{18} column, which had previously been conditioned first with methanol (8 ml) and then with water (8 ml). The elution was carried out in two stages, firstly with water (8 ml) and then with methanol (10 ml). The methanol fraction was evaporated to dryness under reduced pressure and the residue (~ 100 mg) was redissolved in 0.25 ml of methanol, and 2 ml of water containing 1 mg of $KHCO_3$ was added as described above. The saponins in the lupin extract were derivatized in a similar manner to the saponin standards.

5.3.1.2 *High-performance liquid chromatography*

5.3.1.2.a Instrumentation

HPLC analyses were performed on a PU 4100 liquid chromatograph (Pye Unicam) with an autosampler (Gilson, Anachem) coupled to a Philips PU UV 4025 detector and a Gilson 715 data collection system. Separations were performed on a column (25 cm x 4.6 mm ID) packed with Ultratechsphere 5 µ C_{18} (HPLC Technology Ltd, Macclesfield, U.K.).

5.3.1.2.b Chromatographic conditions

Chromatographic runs were carried out with an acetonitrile-water gradient elution system. Solvents, which were degassed with helium, were water (solvent A) and acetonitrile (solvent B). The gradient was run according to the following programme: 58% A for 1 min followed by a reduction to 40% A after 15 min, further decreased to 10% A for 10 min, then held at 10% A over a further 10 min and finally increased to 58% for 10 min. The flow rate, injection volume and run time were 1 ml/min, 10 µl and 46 min respectively. Detection was effected by UV absorption at 260 nm.

5.3.2 High-performance liquid chromatography of underivatized saponins in *L. angustifolius* seed

As mentioned above, Okubo and coworkers have successfully separated, on a qualitative basis using HPLC, intact saponins from several legumes[256-259]. This work has been developed further here for both the separation and quantification of the intact saponins present in lupin seed (*Lupinus angustifolius*).

5.3.2.1 *Mixture of saponin standards*

Soyasaponin I (1 mg), soyasaponin VI (3 mg) and α-hederin (3 mg) were dissolved in butan-1-ol and analyzed by HPLC.

5.3.2.2 *Extraction procedure*

Milled *L. angustifolius* seed (4 g) and α-hederin (3 mg) were firstly soaked in 70% aqueous ethanol containing 0.01% EDTA for 10 min and secondly stirred for 1 h at room temperature. The sample was then centrifuged at 8000 rpm for 15 min at 20°C. This extraction process was repeated three more times and the supernatants combined and filtered through a sinter funnel under reduced pressure. The extract was rotary-evaporated to dryness at less than 40°C and dissolved in a mixture of 2 ml of water and butan-1-ol (1:1, v/v). After centrifugation, the butan-1-ol layer was collected and analyzed by HPLC.

5.3.2.3 *Spectroscopy*

UV scans from 190 to 400 nm of solutions of soyasaponin I, soyasapogenol B, glucuronic acid, soyasaponin VI and α-hederin in ethanol (0.1 mg ml^{-1}) were obtained using a DU-70 spectrophotometer (Beckman) at a speed of 600 nm/min.

5.3.2.4 *High-performance liquid chromatography*

5.3.2.4.a Instrumentation

The instrumentation was the same as that used in Section 5.3.1.2.a.

5.3.2.4.b Chromatographic conditions

Chromatographic runs were carried out with an acetonitrile-water gradient elution system. Solvents were acetonitrile-acetic acid (1000:0.3 v/v) (solvent A) and water:acetic acid:EDTA (1000:0.3:0.15 v/v/w) (solvent B). Solvents were degassed with helium. The gradient was run according to the following programme: 35% A isocratically for 18 min followed by an increase to 42% A after a further 4 min, held at 42% A for 10 min then changed to 35% A over a further 4 min and finally held at 35% A for 8 min. The flow rate, injection volume and run time were 0.9 ml/min, 10 µl and 45 min respectively. Detection was effected by UV absorption at 205 nm.

5.3.3 GC and FAB-MS

These techniques are described in Section 2.3.

5.4 RESULTS AND DISCUSSION

5.4.1 High-performance liquid chromatography of derivatized saponins in *L. angustifolius* seed

Conversion of the saponin into its potassium salt (Figure 5.1) can be easily carried out when it is readily soluble in water. However, both soyasaponin I and soyasaponin VI

(Figure 5.2) possess low solubility in water. This problem was overcome by first dissolving the saponins in a small volume of methanol followed by dropwise addition of potassium hydrogen carbonate solution.

According to Oleszek *et al.*[141], all the saponins present in alfalfa roots could be derivatized via their free carboxylic acid groups. However, those containing only one free carboxylic acid, such as soyasaponin I, had lower responses than those containing two free acid groups. It is also mentioned by these authors that the stability of the 4-bromophenacyl derivatives varied strongly and depended on the structure of the saponin.

Following the guidelines suggested by these authors and bearing in mind the likely instability of the 4-bromophenacyl derivatives of soyasaponins I and VI, the HPLC determination was carried out just after derivatization.

Figures 5.3 shows the chromatogram corresponding to a blank prepared in the same way as for the saponin derivatives (see Section 5.3.1.1) whereas the chromatograms displayed in figures 5.4 and 5.5 correspond to the 4-bromophenacyl derivatives of soyasaponin I and soyasaponin VI, respectively.

The blank mixture was responsible for those peaks with retention times falling between 7 and 17 min (Figure 5.3). The 4-bromophenacyl bromide derivative of soyasaponin I showed a sharp and symmetrical peak (Figure 5.4) at a retention time of 20.6 min. The derivative of soyasaponin VI, however, did not show a well resolved peak (Figure 5.5) and its absence could be attributed to the lack of purity and stability of the sample standard of soyasaponin VI and instability of its derivative. Soyasaponin VI has been reported to be unstable when heated at 100°C for 1 h[256]. Therefore, it is highly probable that this saponin undergoes decomposition during the derivatization process, which involves heating at 100°C for 90 min (see Section 5.3.1.1a).

Figure 5.6 shows the analysis of derivatized saponins present in an extract of lupin (*L. angustifolius*) seed. A comparison of retention times of particular peaks in the mixture with that corresponding to the 4-bromophenacyl derivative of the standard of soyasaponin I allowed the identification of this in the *L. angustifolius* extract.

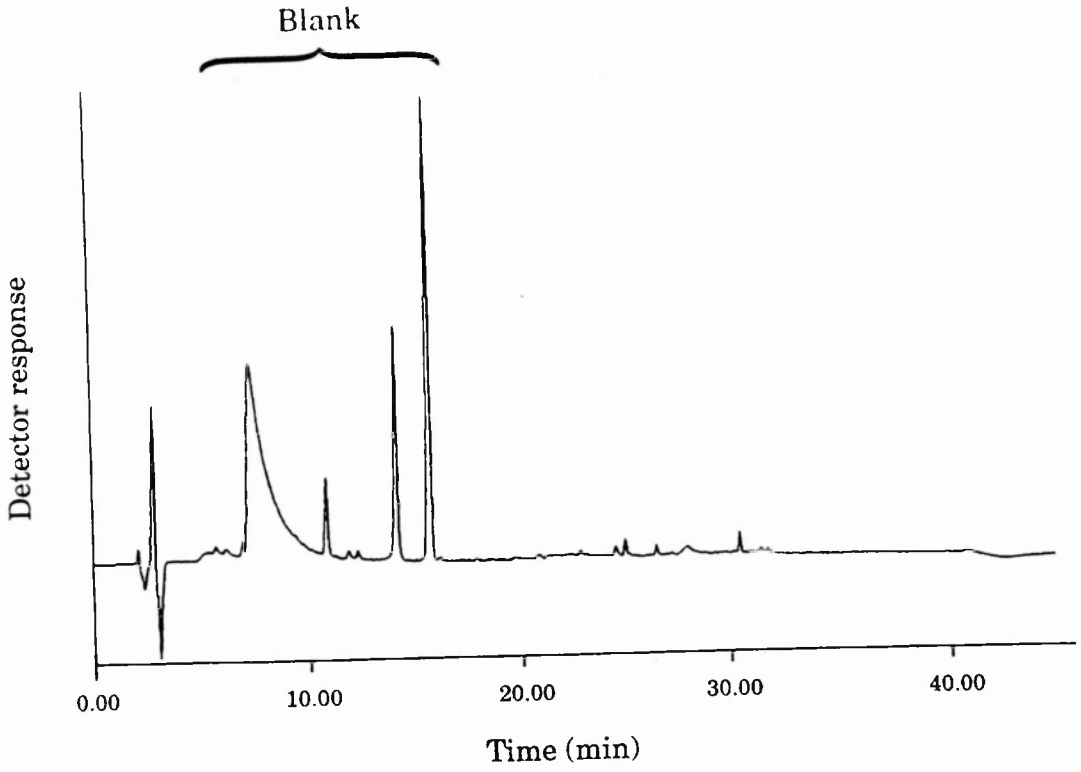


FIGURE 5.3. Chromatogram of a blank containing 4-bromophenacyl bromide and 18-crown-6.

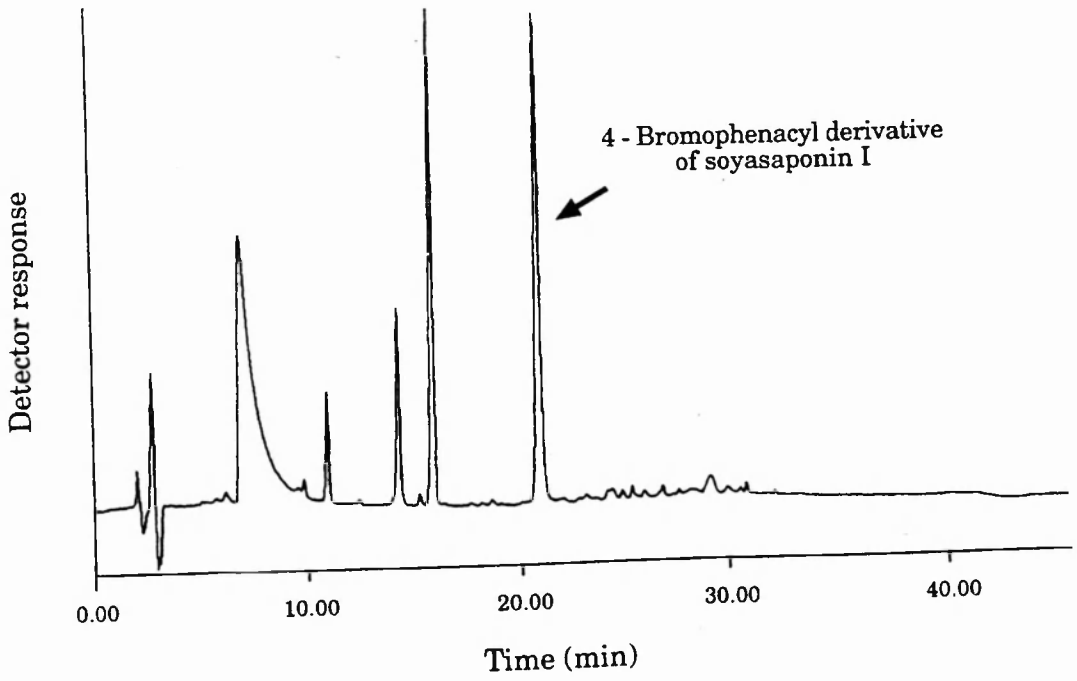


FIGURE 5.4. Chromatogram of the 4-bromophenacyl derivative of soyasaponin I.

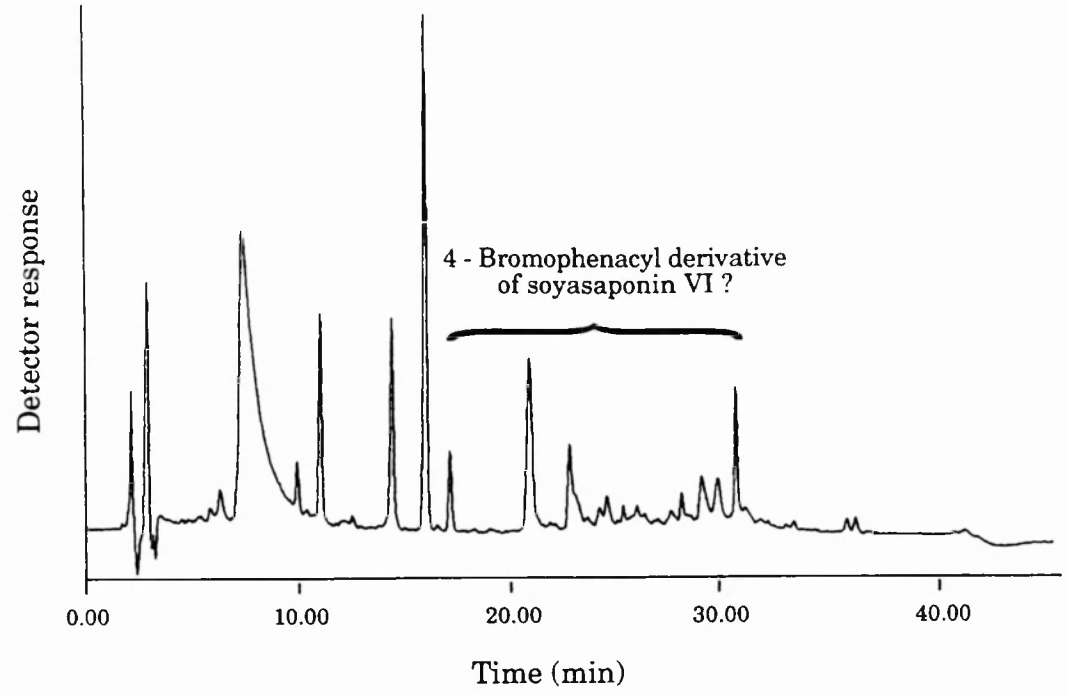


FIGURE 5.5. Chromatogram of 4-bromophenacyl derivative of soyasaponin VI.

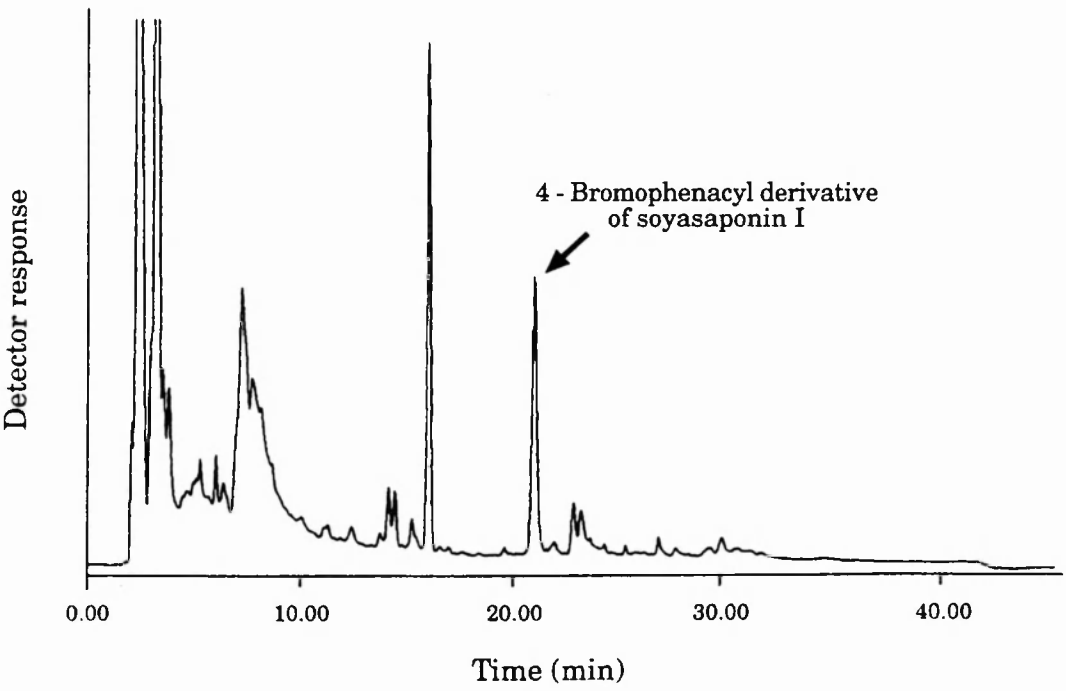


FIGURE 5.6. Chromatogram of derivatized *L. angustifolius* saponins.

In order to test the stability of the 4-bromophenacyl derivative of soyasaponin I, repetitive HPLC determination of the above-mentioned derivative was carried out over a period of 6 h at intervals of 0, 0.8, 2.7, 4, 4.9 and 6.2 h, following derivatisation at room temperature. (Figure 5.7).

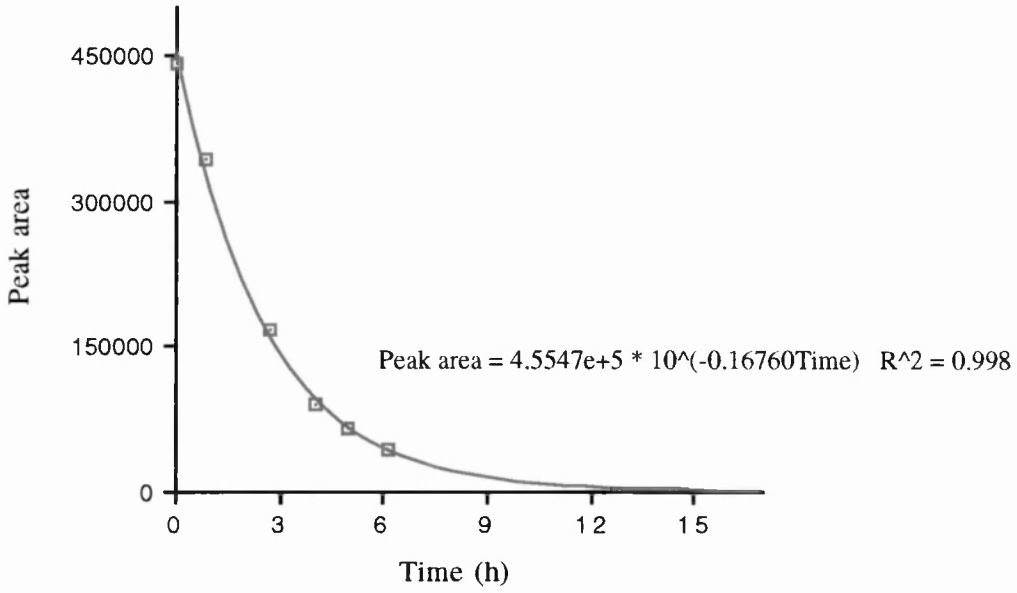


FIGURE 5.7. Decomposition of the 4-bromophenacyl derivative of soyasaponin I.

The decomposition of the 4-bromophenacyl derivative of soyasaponin I fitted an exponential curve with a high correlation coefficient. By extrapolation, complete decomposition would be expected after 15 h, which is not in good agreement with Oleszek *et al.*[141] who found that the above-mentioned derivative of soyasaponin I decomposed after 48 h.

Since soyasaponin VI could not be successfully determined and stability problems with the 4-bromophenacyl derivatives were found, the HPLC method developed by Oleszek *et al.*[141] for alfalfa roots was not considered suitable for the analysis of saponins in legume seed extracts.

5.4.2 High-performance liquid chromatography of underivatized saponins in *L. angustifolius* seed*

UV scans for soyasaponin I (Figure 5.8) and soyasapogenol B (Figure 5.9) showed a maximum absorption at 205 nm while 212 nm was the maximum detected for glucuronic acid (Figure 5.10). Soyasaponin VI (Figure 5.11) displayed maxima at 205 and 292 nm, as expected. Therefore, both saponins can be measured by their UV absorption at 205 nm which arises from the carbon-to-carbon double bond in the triterpenoid aglycone although a small contribution to it stems from the carboxylic group in the glucuronic acid (Figures 5.8, 5.9 and 5.10).

HPLC analyses of soyasaponin I and soyasaponin VI (Figure 5.2) together with the internal standard α-hederin, which is a commercial saponin extracted from ivy also with a maximum absorption at 205 nm (Figure 5.12), revealed well resolved peaks whose retention times were 16.0, 28.3 and 32.3 min respectively (see Figure 5.13).

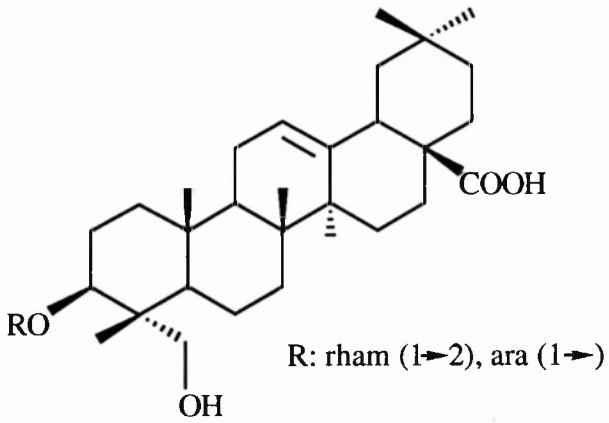


FIGURE 5.12. Chemical structure of α-hederin.

* The data in this section have been published in :

Ruiz, R.G., Price, K.R., Rose, M.E., Rhodes, M.J.C. and Fenwick, G.R. (1995). *J. Liq. Chromatog.*, **18**: 2843-2853.

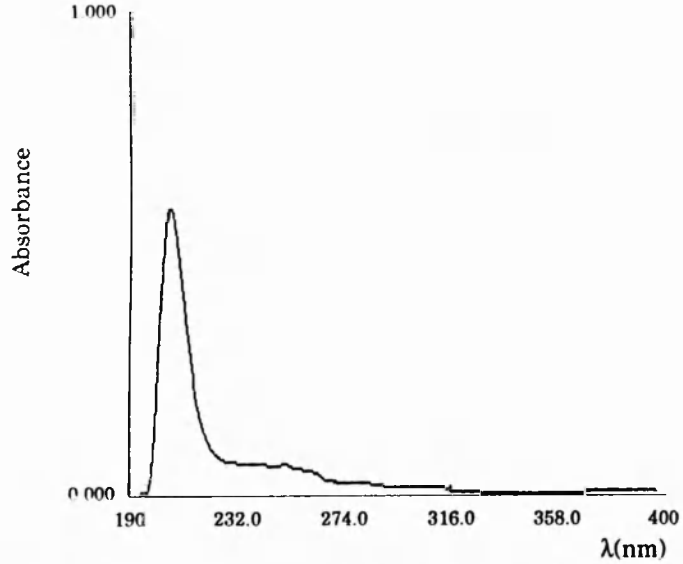


FIGURE 5.8. UV spectrum of soyasaponin I.

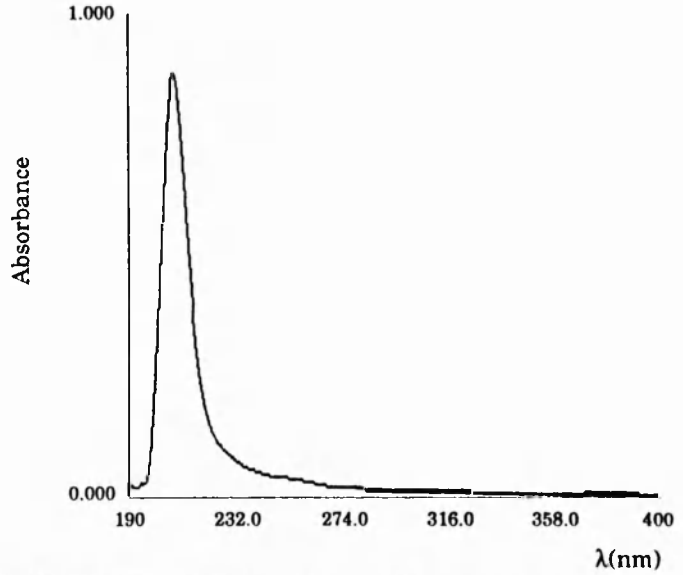


FIGURE 5.9. UV spectrum of soyasapogenol B.

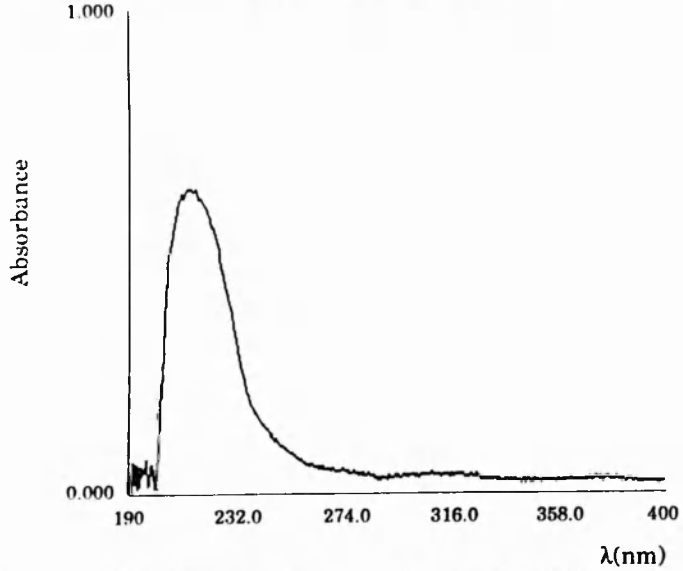


FIGURE 5.10. UV spectrum of glucuronic acid.

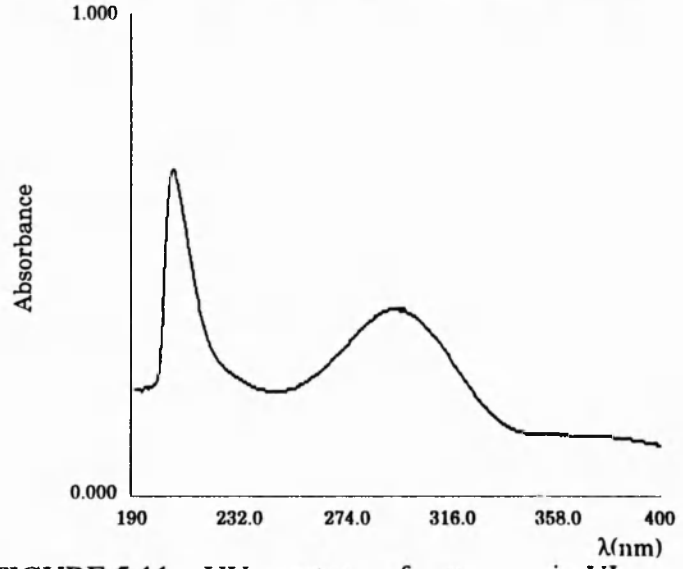


FIGURE 5.11. UV spectrum of soyasaponin VI.

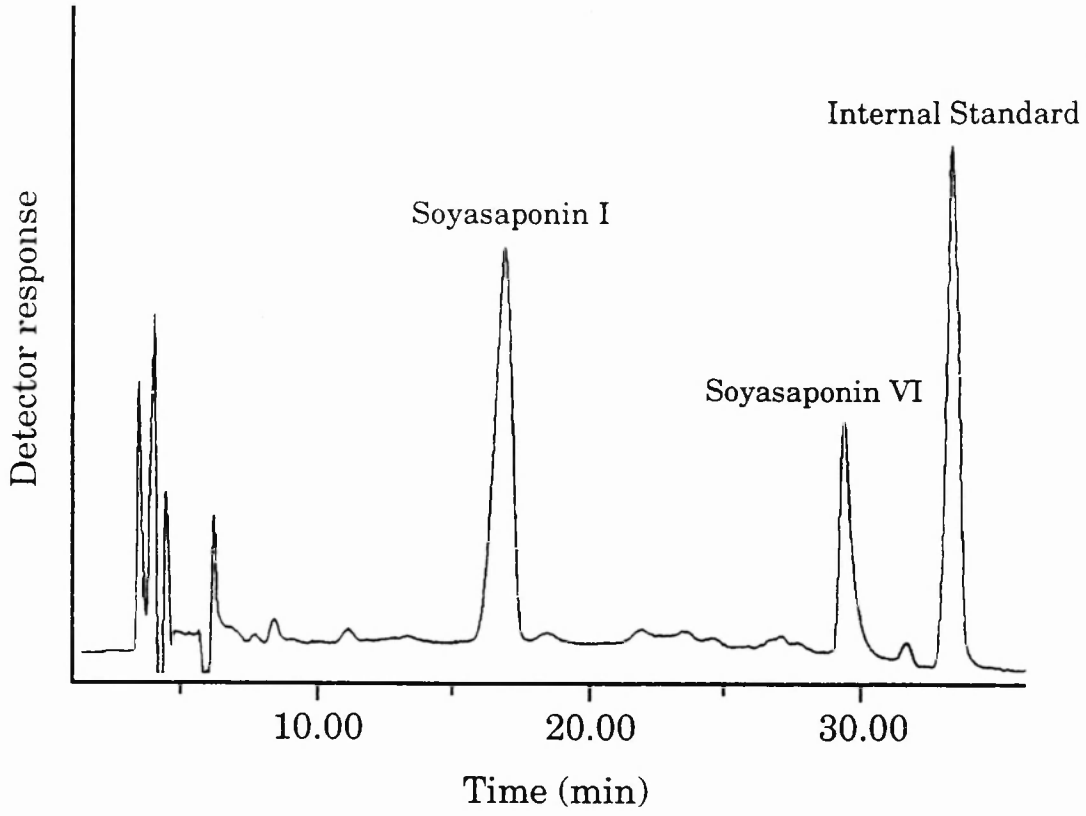


FIGURE 5.13. Chromatogram of a mixture of soyasaponin I, soyasaponin VI and α-hederin.

Due to the relative instability of soyasaponin VI and in order to check its purity, data from the GC analysis of soyasapogenol B, which resulted from the hydrolysis of 1 mg of each of soyasaponin I and soyasaponin VI, showed soyasaponin VI to be 30% pure.

Peak-area ratio calibrations with the internal standard (HPLC) were then constructed for both soyasaponin I and soyasaponin VI with the purity of soyasaponin VI calculated from GC measurements being included in these calculations. The equation constant, correlation coefficient, weight range and limit of detection for each saponin are illustrated in Table 5.1. The calibrations plots showed good linearity for both soyasaponin I and soyasaponin VI (see Figure 5.14).

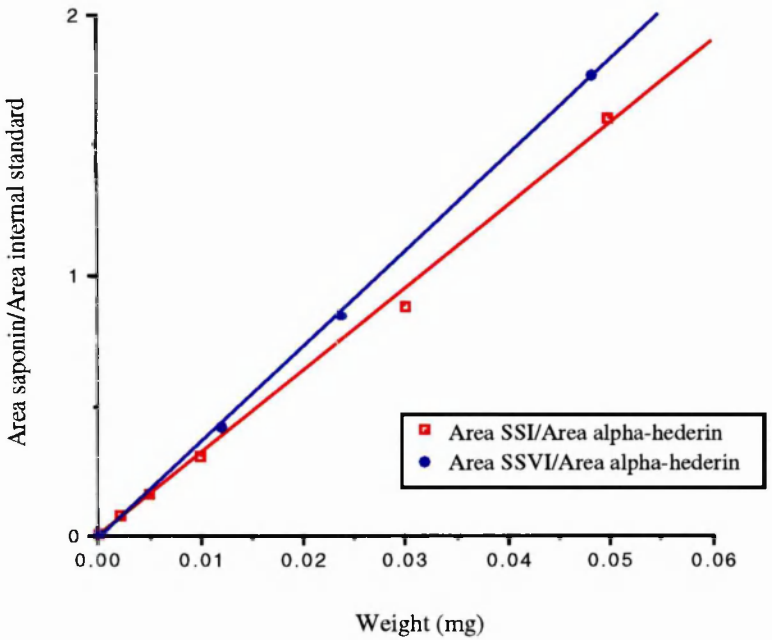


FIGURE 5.14. Calibration curves for soyasaponin I (SSI) and soyasaponin VI (SSVI).

TABLE 5.1. Chromatographic constants for HPLC analysis.

Chromatographic Constants	**Soyasaponin I (SSI)**	**Soyasaponin VI (SSVI)**
Equation y = a + bx		
a	$-4.9228e^{-3}$	$-1.4656e^{-2}$
b	$3.1573e^{-2}$	$3.6615e^{-2}$
r	0.998	1.000
Range (µg)	0 - 50	0 - 48.3
Internal Standard (µg)	30	30
Limit of Detection (µg)*	3.2	1.5

*The limit of detection was calculated as the weight (µg) of saponin injected which gave a signal three times greater than the 'background' noise.

FAB-MS of the seed extract containing the intact saponins after exhaustive hot solvent extraction as described in Section 2.3.1, showed mainly the presence of soyasaponins I and VI. The total saponin content of the *L. angustifolius* seed was estimated to be 385 mg/kg by quantifying the acid-released sapogenol from GC analysis.

In contrast the chromatogram resulting from the analysis of the *L. angustifolius* seed extract by HPLC following the mild extraction conditions described in Section 5.3.2.2 is shown in Figure 5.15. Retention time comparisons relative to the internal standard α-hederin and confirmation by co-chromatography of the reference saponins showed the presence of only soyasaponin VI. Soyasaponin I was not detected.

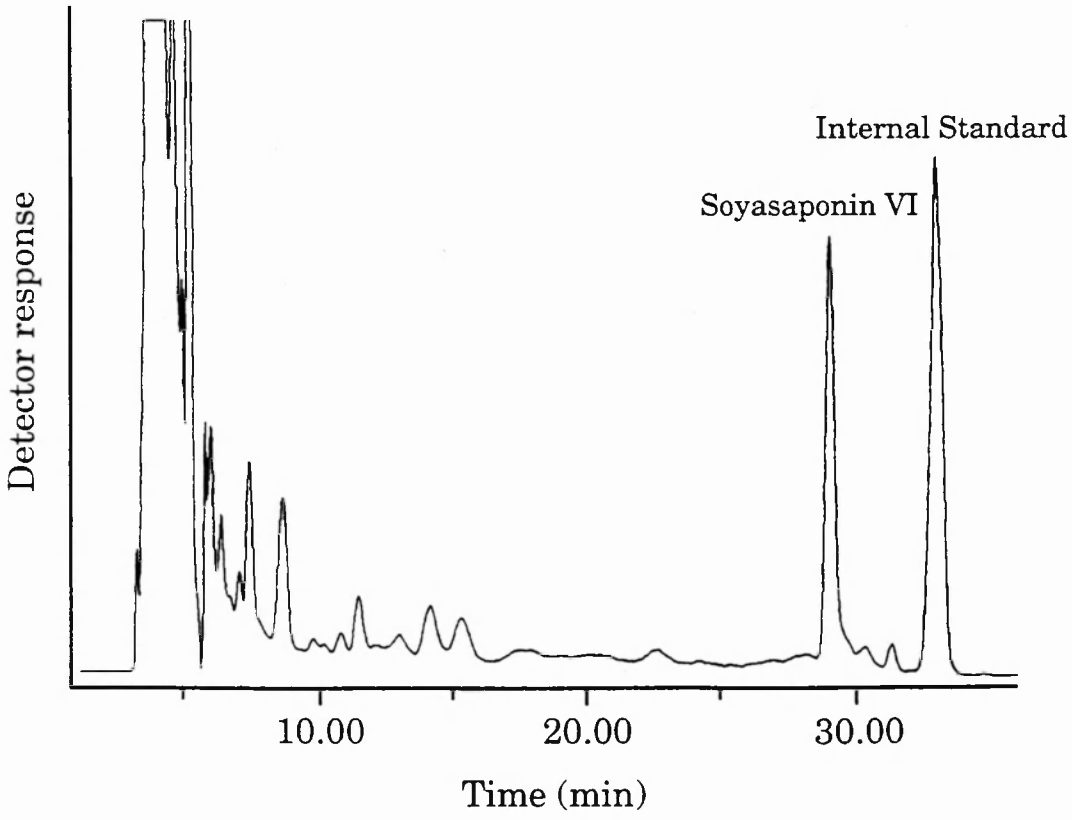


FIGURE 5.15. Chromatogram of *L. angustifolius* seed saponins (soyasaponin VI) with internal standard (α-hederin).

The saponin content of the *L. angustifolius* seed was estimated to be 450 mg/kg using the HPLC technique. In order to test any possible partition of saponins between the water and the butan-1-ol layers in which the dried ethanol extract was dissolved prior to HPLC analysis of latter layer (Section 5.3.2.2), the water layer was also collected and analyzed by HPLC. No saponin was detected.

The saponin content of the lupin seed by HPLC was 14% higher than that obtained from the GC technique, which suggests that saponin losses occurred during some of the stages involved in the latter method. Since the internal standard used for the GC analysis (cholesteryl-n-decanoate) is not glycosidic in nature it cannot be added during the extraction, cleanup or hydrolysis stages and it is likely therefore that these would be the stages where saponin loss could occur.

The extraction of saponins involved in the HPLC method described here was performed four times in aqueous ethanol at room temperature (Section 5.3.2.2). The efficiency of this extraction procedure was examined by HPLC. The third and fourth extraction steps were shown to provide only a limited contribution to the total saponin content of the sample. After a fifth extraction step, no saponin was detected. The mild extraction technique was also confirmed to be quantitative by subsequent exhaustive hot solvent extraction as described in Section 2.3.1 and HPLC analysis of the extract in which neither soyasaponin I nor soyasaponin VI was detected.

The fact that soyasaponin VI was the only saponin detected in the lupin seed extract by HPLC after mild solvent extraction is in agreement with Massiot *et al.*[254] who isolated soyasaponin VI from alfalfa and characterized it as the natural precursor of soyasaponin I and Kudou *et al.*[256] who postulated that DDMP-conjugated saponins were, in fact, the genuine saponins in the case of native soyabean seeds whilst soyasaponins I-V, the saponins normally associated with soya following conventional exhaustive hot solvent extraction, were in fact artifacts derived from degradation of the DDMP saponins during the extraction or concentration processes.

The HPLC method described here has obvious advantages over the one

developed by Oleszek *et al.* [141] since it provides the quantification of intact, underivatised saponins in legumes and it does not suffer from stability and selectivity problems. This HPLC method for the analysis of saponins in legumes also has several advantages over the one described by Okubo and coworkers[256-259]. First of all, quantification of individual saponins has been achieved with the use of a suitable internal standard, which is added before extraction; therefore, if saponin losses occurred during the extraction or concentration procedures, both saponins and internal standard would be affected on an equal basis. Secondly, since saponins are monitored by UV absorption at 205 nm, it will allow the fate of soyasaponin VI, which has been shown to be relatively unstable, to be determined after industrial or domestic processing. If partial or total conversion of soyasaponin VI into its breakdown product, soyasaponin I, arises from such processing, both detection and quantification of either of these two saponins with the internal standard will be easily achieved at 205 nm. Thirdly, the use of a gradient elution system in the HPLC method described here has resulted in a shorter chromatographic run time compared to the isocratic elution used by Okubo and coworkers[256-259].

5.5 CONCLUSIONS

- An HPLC method for the quantification of intact, underivatised individual saponins in *Lupinus angustifolius* seed with the use of a suitable internal standard and a proven mild quantitative extraction technique have been developed.

- The saponin content of the *L. angustifolius* seed was estimated to be 450 mg/kg using the new developed HPLC method. Soyasaponin VI, a DDMP-conjugated form of soyasaponin I, was the only saponin detected in the lupin seed.

- The technique has been shown to have several advantages over three other

published methods; (a) the conventional GC method, which quantifies the sapogenol resulting from the acid hydrolysis of the saponins, (b) a HPLC method for the analysis of derivatized saponins and (c) a HPLC method for the qualitative determination of intact saponins.

- This new HPLC method will enable quantitative studies to be made for the first time on the effect of different kinds of processing not only on the total saponin content but also on the saponin composition.

VI Effect of Processing on the Saponin Content and Composition of Chickpea and Lentil Seed

6.1 INTRODUCTION

Both chickpeas and lentils are leguminous crops that are used in various forms, mainly for human consumption, and that constitute excellent sources of protein, carbohydrates, fibre, minerals, and other nutrients. However, several antinutritional factors are found in biologically significant amounts in the raw seeds of these legumes (see Sections 1.1.3.3 and 1.1.4.3).

Among the naturally occurring compounds of grain legumes, saponins have been shown to possess diverse properties, both deleterious and beneficial. Fungitoxic[204-208], haemolytic[36,37], and membranolytic[182-184] activities have been ascribed to saponins. Conversely, a beneficial lowering of plasma cholesterol levels in humans[234] has also been attributed to saponins whilst some have been reported to exhibit anticancer activity[237-241] and inhibitory effect on the infectivity of HIV *in vitro*[199](see Section 1.2.9).

Legumes normally used in human nutrition need to be processed prior to consumption to reduce their levels of antinutritional factors. The traditional domestic methods of legume processing include milling, soaking, germination, fermentation, cooking and toasting. Of these, soaking, cooking and germination are the processes most commonly used.

Soaking of food legumes usually forms an integral part of bean processing methods such as cooking, germination and fermentation. It involves the moisturizing of the seeds by the soaking solution penetrating their physical structure. The first observed effects are an increase in the water content of the seed and the diffusion of soluble components[275]. The effect of this treatment on the elimination of antinutritional factors depends on the contribution of each of the following factors: type and volume of soaking solution, soaking duration, temperature, exposure to light, compound stability and elimination of liquid after processing. The soaking solutions most commonly used are water and dilute saline and alkali solutions. Both salts and alkalis help diffusion of

soluble compounds into the soaking solution by increasing the permeability of cell membranes. The soaking solution is important in order to achieve a good texture, reduce cooking times and provide legumes with better sensory properties[275,276].

Soaking of lentils in either distilled water, citric acid or sodium bicarbonate solutions decrease phytic acid content, increase tannins and catechins, and has no effect on trypsin inhibitor activity[277]. Soaking was also found to reduce drastically the alpha-galactoside content of lentils[278,279] and chickpeas[280].

Cooking is probably the oldest known method of bean processing. The cooking process softens hard seed by improving the plasticity of the cell wall and gelatinization of the starch. Legumes require between 30 and 90 min boiling to be tender and ready for consumption. This cooking time can be reduced depending on additional factors such as soaking prior to boiling, addition of salt to both the soaking and boiling solutions and the boiling temperature which can be modified by changes in pressure[275]. Cooking develops the flavour of the seeds and the cooking broth may or may not discarded, depending upon cultural and personal preferences[281]. If both the soaking and cooking solutions have not been removed, heat-stable antinutritional factors such as estrogens and allergens may not be reduced significantly after the heat processing[275].

Cooking reduced to a large extent the trypsin inhibitor activity of chickpeas[282] and totally removed the activity in lentils whilst reducing phytic acid and increasing tannins and catechins[277]. A considerable decrease in the amount of the raffinose oligosaccharides in chickpeas[280] and lentils[278] was also observed after combined soaking and cooking treatment.

Legume sprouts constitute a significant proportion of the total consumption of food legumes, particularly in the Far East[283]. Germinated dry beans are receiving increasing attention because of the probability that flavour and nutritional qualities are improved and in recent years, legume sprouts have been used in the preparation of legume-based, low-cost weaning foods[284]. Germination mobilizes reserve nutrients

required for growth and therefore may help removal of some of the unwanted components of legume seeds which are thought to function as reserve nutrients (e.g. phytates and raffinose oligosaccharides).

A total elimination of raffinose oligosaccharides[285] and a large decrease in the phytic acid content of lentils were observed after 6 days of germination[285,287]. Germination also lowered trypsin inhibitor activity in lentils, and increased the amount of tannins and catechins[277]. In germinated chickpeas, a significant reduction in raffinose and stachyose[286] and trypsin inhibitor activity was observed[286,287].

The effects of soaking, cooking and sprouting on saponins are poorly understood and there is clearly a need to examine in detail the effects on both saponin composition and content of such processing since preliminary studies have demonstrated a relationship between chemical structures and biological activity[185-187,259].

The method for the quantitative determination of intact DDMP saponins in *Lupinus angustifolius* seed by high-performance liquid chromatography described in Section 5.3.2 has now been used to study the effect of soaking (using distilled water, citric acid and bicarbonate solutions), cooking for 30, 60, 90 and 120 min, after the seeds were presoaked in distilled water, and germination in the dark for 6 days at 20°C on the saponin content and composition of two cultivars of both chickpeas and lentils.

6.2 MATERIALS

Two cultivars of both chickpeas (*Cicer arietinum*), Fardon (type: *Desi*) and Blanco Lechoso (type: *Kabuli*) (Figure 6.1), and lentils (*Lens culinaris*), Magda-20 (type: *castellana*, ssp: *macrosperma*) and Lyda (type: *castellana*, ssp: *macrosperma*)(Figure 6.2), were obtained from crops grown in Córdoba and Albacete (Spain), respectively.

A standard sample of soyasaponin VI, also called ßg, was kindly donated by Dr. K. Okubo and Dr. Y. Yoshiki (Tohoku University, Japan). Soyasaponin I was available

from previous work[95]. α-Hederin was purchased from Apin Chemicals Ltd., Abingdon, Oxon, U.K.

All solvents used during the extraction process were of analytical-reagent grade. Acetonitrile, ethanol, butan-1-ol, acetic acid and sodium hydrogen carbonate were purchased from BDH Laboratory Supplies, Poole, U.K. Ethylenediaminetetraacetic acid (EDTA) and citric acid were supplied by Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, U.K. Water was purified using a Milli-Q system, Waters Chromatography Division, Millipore Corporation, Milford, U.S.A.

FIGURE 6.1. Seed from the two cultivars of Spanish chickpeas.

FIGURE 6.2. Seed from the two cultivars of Spanish lentils.

6.3 METHODS

6.3.1 Pretreatment of the seeds

Seeds of a similar size were selected and placed in a vacuum desiccator with silica gel for 92 h to achieve a relative constant weight with regard to the moisture content of the seeds.

6.3.2 Processes

6.3.2.1 *Soaking*

This process was performed in three types of solutions: distilled water, 0.1% (0.1g/100

ml) citric acid, and 0.07% (0.07g/100 ml) sodium bicarbonate. The proportion of seed to soaking medium was 1:3 w/v. The soaking period, 24 h at 25 °C, was chosen to obtain maximum seed weight and hydration. The soaking solution was drained and both the soaking solutions and the soaked seeds were freeze-dried and the latter milled.

The initial seed weight (after having been placed in a vacuum desiccator for 92 h), the seed weight after draining the soaking solution, and the combined weight of seed plus solution after freeze-drying are listed in Tables 6.1 and 6.2 for chickpeas and lentils respectively. Also shown in these tables are volumes, dry weights and pH of the soaking solutions both before and after the different treatments for both varieties of legumes.

The soaking process for both chickpeas and lentils has been summarized in Figure 6.3.

6.3.2.1.a Hydration coefficient

The hydration coefficient of both chickpeas and lentils, which were soaked in distilled water, 0.1% citric acid and 0.07% sodium bicarbonate solutions for 24 h at 25°C, was determined according to the following formula[288]:

Hydration Coefficient (%) = (weight of seed after soaking / initial weight) x 100

TABLE 6.1. Table of weights of seeds before and after soaking, dry weights of both seeds and soaking solutions, and volumes and pHs of the latter, before and after soaking, for both cultivars of chickpeas.

Cultivar and Treatment	*Before Soaking*			*After Soaking*			*Dry Weight*[b]		
	Weight seeds[a] *(g)*	*Volume solution (ml)*	*pH solution*	*Weight seeds (g)*	*Volume solution (ml)*	*pH solution*	*Seeds (g)*	*Solution (g)*	*Total (g)*
Chickpeas Fardon									
unprocessed	200						189		189
24 h water soaking	200	600	6.26 ± 0.02	435	360	6.25 ± 0.02	177	6	183
24 h citric acid soaking	200	600	2.79 ± 0.02	418	370	5.53 ± 0.02	175	9	184
24 h s. bicarbonate soaking	200	600	8.83 ± 0.02	429	360	6.60 ± 0.02	178	6	184
Chickpeas Blanco Lechoso									
unprocessed	200						189		189
24 h water soaking	200	600	6.26 ± 0.02	454	335	6.24 ± 0.02	182	3	185
24 h citric acid soaking	200	600	2.79 ± 0.02	451	330	5.29 ± 0.02	180	5	185
24 h s. bicarbonate soaking	200	600	8.83 ± 0.02	458	330	6.59 ± 0.02	181	4	185

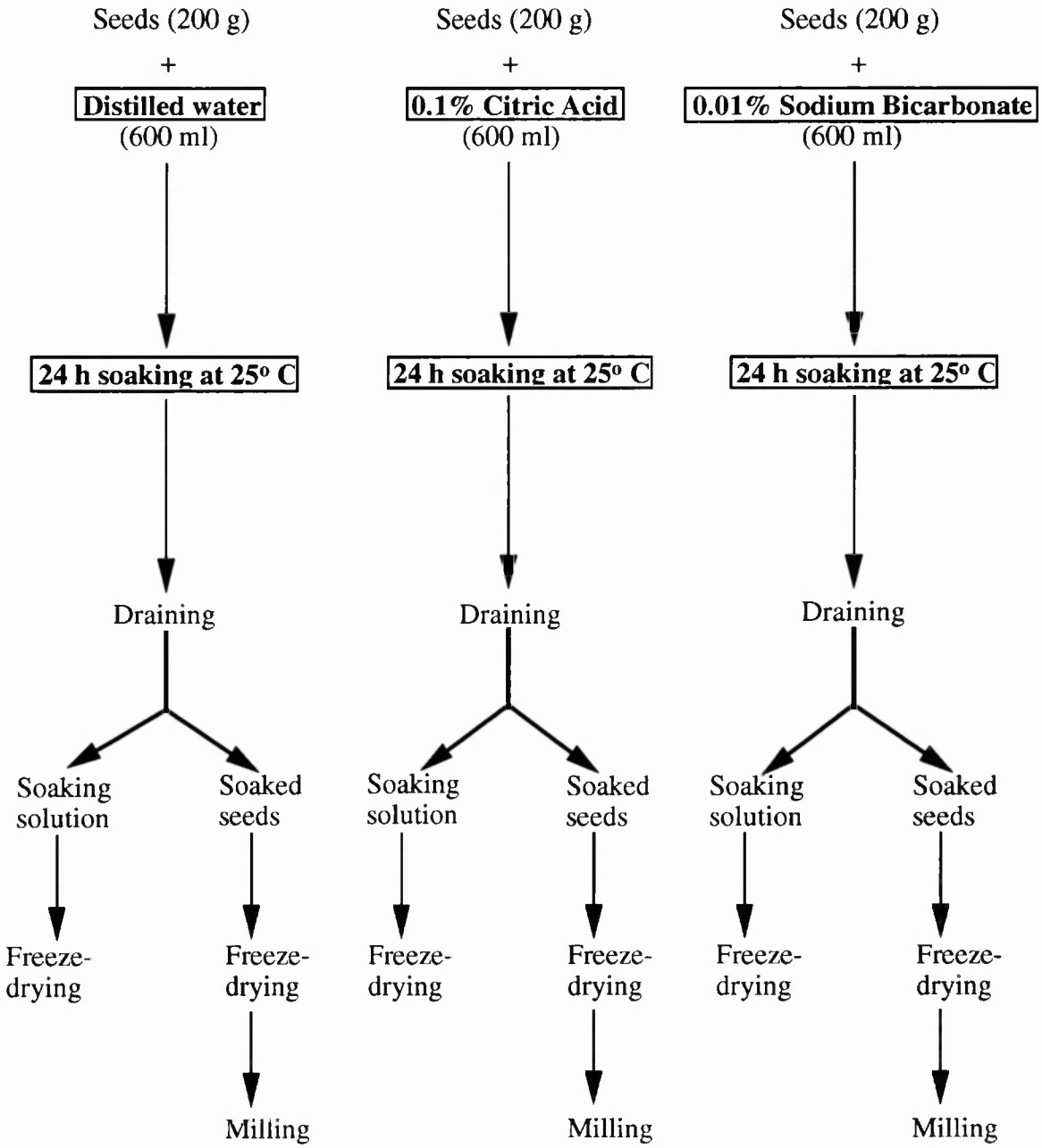
[a] weight of the seeds after having been in a vacuum desiccator for 92 h.
[b] weight of both seeds and soaking solutions after freeze-drying.

TABLE 6.2. Table of weights of seeds before and after soaking, dry weights of both seeds and soaking solutions, and volumes and pHs of the latter, before and after soaking, for both cultivars of lentils.

Cultivar and Treatment	*Before Soaking*			*After Soaking*			*Dry Weight*[b]		
	Weight seeds[a] *(g)*	*Volume solution (ml)*	*pH solution*	*Weight seeds (g)*	*Volume solution (ml)*	*pH solution*	*Seeds (g)*	*Solution (g)*	*Total (g)*
Lentils Magda 20									
unprocessed	200						192		192
24 h water soaking	200	600	6.05 ± 0.02	470	330	6.42 ± 0.02	179	6	185
24 h citric acid soaking	200	600	2.75 ± 0.02	464	330	5.73 ± 0.02	179	6	185
24 h s. bicarbonate soaking	200	600	8.45 ± 0.02	460	330	6.64 ± 0.02	179	6	185
Lentils Lyda									
unprocessed	200						187		187
24 h water soaking	200	600	5.91 ± 0.02	425	365	6.15 ± 0.02	180	3	183
24 h citric acid soaking	200	600	2.77 ± 0.02	415	365	4.72 ± 0.02	179	4	183
24 h s. bicarbonate soaking	200	600	8.79 ± 0.02	421	365	6.24 ± 0.02	181	3	184

[a] weight of the seeds after having been in a vacuum desiccator for 92 h.
[b] weight of both seeds and soaking solutions after freeze-drying.

FIGURE 6.3. Soaking treatments for both chickpeas and lentils.



6.3.2.2 *Soaking plus cooking*

Four portions of seeds were soaked in distilled water for 12 h at 25 °C (seed:water ratio 1:3 w/v). The soaking solution was drained and the soaked seeds were boiled for 30, 60, 90 and 120 min respectively in distilled water using a teflon-coated iron saucepan. The proportion of seed to water was 3.2, 3.1, 3.1 and 3.3 w/v for chickpeas Fardon, chickpeas Blanco Lechoso, lentils Magda 20 and lentils Lyda, respectively. Approximately 10 min passed before the boiling point was reached in every case. The cooking liquids and seeds were separated using a strainer and freeze-dried. The latter were then ground to a powder.

Figures 6.4 and 6.5 show lentils Lyda before and after the soaking plus cooking processes.

FIGURE 6.4. Lentils Lyda before and after the soaking plus cooking processes.

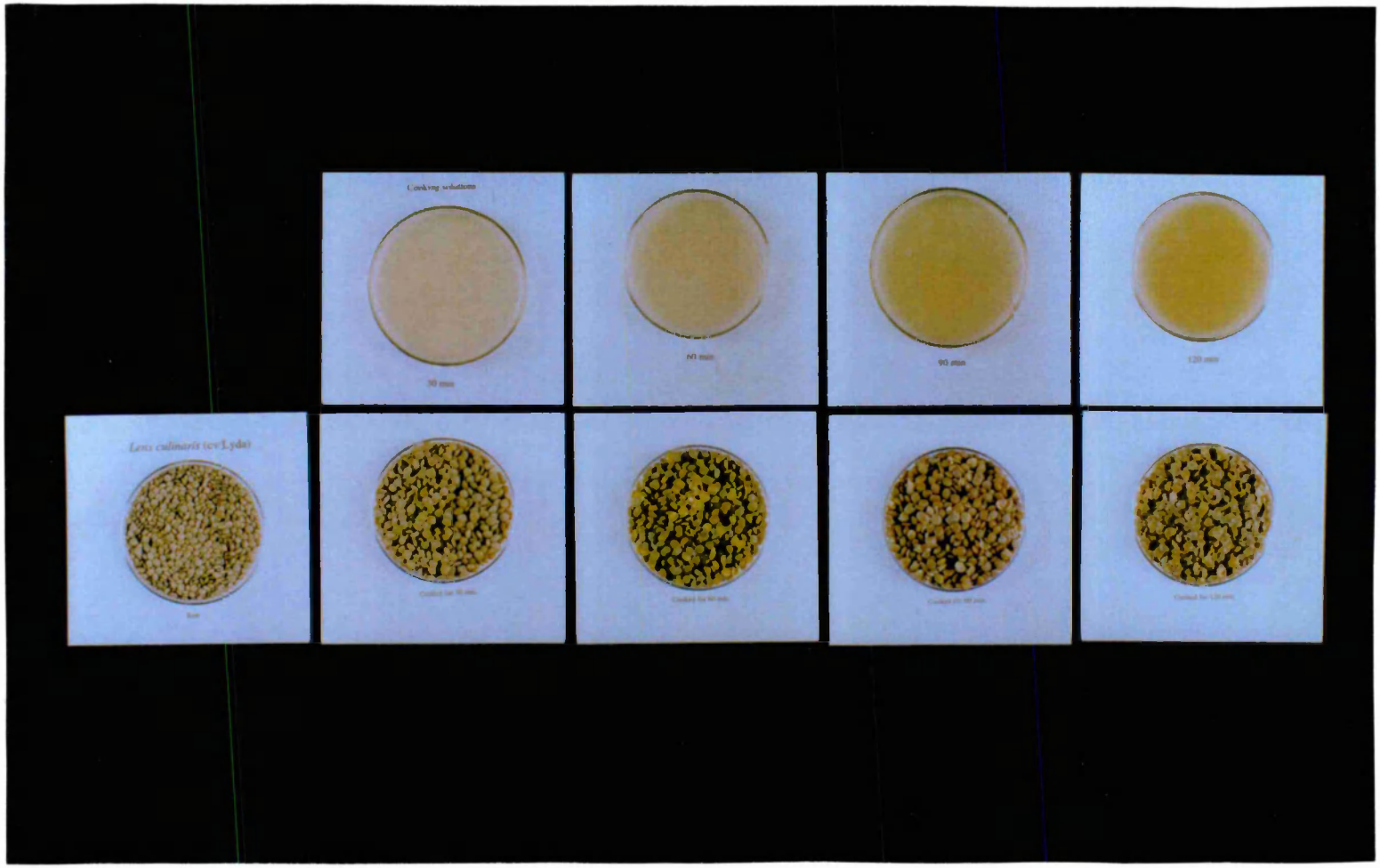


FIGURE 6.5. Lentils Lyda before and after the soaking plus cooking processes.

The initial weight of the seeds, the weight of the seeds after draining the soaking and cooking solutions, and the weights of both seeds and cooking solutions after having been freeze-dried together with the volumes and pHs of the solutions before and after the soaking and cooking processes are shown in Tables 6.3 and 6.4.

The whole soaking plus cooking process for both chickpeas and lentils is displayed in Figure 6.6.

6.3.2.2.a Hydration coefficient

The hydration coefficient of both chickpeas and lentils, which were soaked in distilled water for 12 h at 25°C prior to cooking, was calculated as in Section 6.3.2.1.a.

6.3.2.2.b Microscopic examination

Small pieces of both unprocessed and 30 min-cooked chickpeas Fardon and lentils Lyda were fixed in 3% glutaraldehyde in 0.05 M cacodylate buffer (pH: 7.4) for 12 h. After fixation, the samples were dehydrated in an ethanol series from 10% to 100% over one day. They were then infiltrated and embedded in acrylic resin (LR white) by polymerising for 24 h at 60°C, and very thin sections (1-2 μm thickness) were cut using an ultramicrotome (Reichert Ultracut E). The sections from the unprocessed seeds were stained with 1% toluidine blue in 1 % borax (pH: 11), which is a metachromic dye that shows proteins blue but does not stain starch. The sections corresponding to the cooked seeds were stained first with 1% toluidine blue and then with 1% iodine in potassium iodide, which is specific for starch.

Microscope examination was carried out using a Leitz Ortholux microscope and photographs (x 480) were taken of both the unprocessed and cooked seeds.

TABLE 6.3. Table of weights of seeds before and after soaking and cooking, dry weights of both seeds and cooking solutions, and volumes and pHs of solutions, before and after soaking and cooking, for both cultivars of chickpeas.

	Before Soaking			*After Soaking*			*Before Cooking*		*After Cooking*			*Dry Weight*[c]		
Cultivar and Treatment	*Weight seeds*[a] *(g)*	*Volume solution*[b] *(ml)*	*pH solution*[b]	*Weight seeds (g)*	*Volume solution (ml)*	*pH solution*	*Volume solution*[b] *(ml)*	*pH solution*[b]	*Weight seeds (g)*	*Volume solution (ml)*	*pH solution*	*Seeds (g)*	*Solution (g)*	*Total (g)*
Chickpeas Fardon														
• unprocessed	100											95		95
• 12 h water soaking plus 30 min cooking in water	100	300	6.35 ± 0.02	219	180	6.23 ± 0.02	700	6.35 ± 0.02	266	595	6.18 ± 0.02	83	8	91
• 12 h water soaking plus 60 min cooking in water	100	300	6.35 ± 0.02	219	180	6.25 ± 0.02	700	6.35 ± 0.02	253	580	6.16 ± 0.02	80	9	89
• 12 h water soaking plus 90 min cooking in water	100	300	6.35 ± 0.02	219	180	6.22 ± 0.02	700	6.35 ± 0.02	252	570	6.14 ± 0.02	79	11	90
• 12 h water soaking plus 120 min cooking in water	100	300	6.35 ± 0.02	219	180	6.26 ± 0.02	700	6.35 ± 0.02	246	550	6.17 ± 0.02	78	11	89
Chickpeas Blanco Lechoso														
• unprocessed	100											93		94
• 12 h water soaking plus 30 min cooking in water	100	300	6.25 ± 0.02	225	169	6.13 ± 0.02	700	6.30 ± 0.02	267	620	6.12 ± 0.02	82	8	90
• 12 h water soaking plus 60 min cooking in water	100	300	6.25 ± 0.02	224	171	6.17 ± 0.02	700	6.30 ± 0.02	257	580	6.09 ± 0.02	79	10	89
• 12 h water soaking plus 90 min cooking in water	100	300	6.25 ± 0.02	225	170	6.14 ± 0.02	700	6.30 ± 0.02	252	540	6.11 ± 0.02	78	12	90
• 12 h water soaking plus 120 min cooking in water	100	300	6.25 ± 0.02	225	170	6.15 ± 0.02	700	6.30 ± 0.02	244	500	6.08 ± 0.02	77	13	90

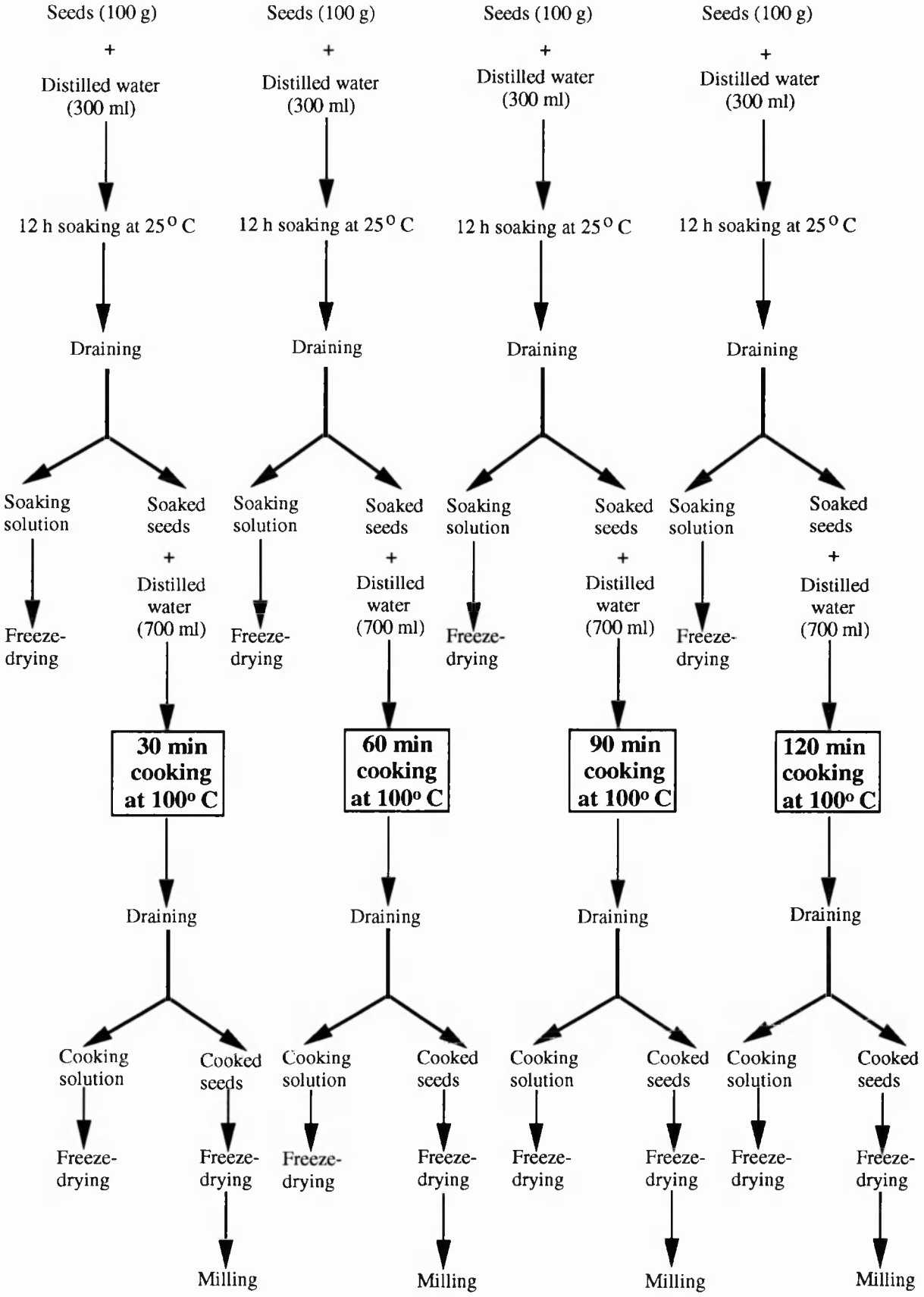
[a] weight of the seeds after having been in a vacuum desiccator for 92 h; [b] distilled water; [c] weight of both seeds and cooking solutions after freeze-drying.

TABLE 6.4. Table of weights of seeds before and after soaking and cooking, dry weights of both seeds and cooking solutions, and volumes and pHs of solutions, before and after soaking and cooking, for both cultivars of lentils.

	Before Soaking			*After Soaking*			*Before Cooking*		*After Cooking*			*Dry Weight*[c]		
Cultivar and Treatment	*Weight seeds*[a] *(g)*	*Volume solution*[b] *(ml)*	*pH solution*[b]	*Weight seeds (g)*	*Volume solution (ml)*	*pH solution*	*Volume solution*[b] *(ml)*	*pH solution*[b]	*Weight seeds (g)*	*Volume solution (ml)*	*pH solution*	*Seeds (g)*	*Solution (g)*	*Total (g)*
Lentils Magda 20														
• unprocessed	100											96		96
• 12 h water soaking plus 30 min cooking in water	100	300	6.07 ± 0.02	224	175	6.02 ± 0.02	700	6.10 ± 0.02	344	460	6.17 ± 0.02	75	15	90
• 12 h water soaking plus 60 min cooking in water	100	300	6.07 ± 0.02	224	175	6.01 ± 0.02	700	6.10 ± 0.02	342	435	6.14 ± 0.02	76	14	90
• 12 h water soaking plus 90 min cooking in water	100	300	6.07 ± 0.02	225	175	6.01 ± 0.02	700	6.10 ± 0.02	335	405	6.15 ± 0.02	75	14	89
• 12 h water soaking plus 120 min cooking in water	100	300	6.07 ± 0.02	225	175	6.03 ± 0.02	700	6.10 ± 0.02	335	395	6.13 ± 0.02	75	15	90
Lentils Lyda														
• unprocessed	100											94		94
• 12 h water soaking plus 30 min cooking in water	100	300	6.15 ± 0.02	211	188	6.11 ± 0.02	700	6.15 ± 0.02	366	435	6.20 ± 0.02	80	11	91
• 12 h water soaking plus 60 min cooking in water	100	300	6.15 ± 0.02	212	186	6.10 ± 0.02	700	6.15 ± 0.02	357	410	6.18 ± 0.02	80	11	91
• 12 h water soaking plus 90 min cooking in water	100	300	6.15 ± 0.02	213	187	6.08 ± 0.02	700	6.15 ± 0.02	354	405	6.17 ± 0.02	79	11	90
• 12 h water soaking plus 120 min cooking in water	100	300	6.15 ± 0.02	212	188	6.09 ± 0.02	700	6.15 ± 0.02	354	400	6.19 ± 0.02	78	13	91

[a] weight of the seeds after having been in a vacuum desiccator for 92 h; [b] distilled water; [c] weight of both seeds and cooking solutions after freeze-drying.

FIGURE 6.6. Cooking treatment for both chickpeas and lentils.



6.3.2.3 *Germination*

Seeds (10 g), after having been in a vacuum desiccator for 92 h, were soaked in 50 ml of distilled water at room temperature and shaken every 30 min. After 6 h, the water was drained off and the seeds were transferred to a separating funnel and kept in the dark at 20°C to germinate for 6 days. Every 24 h the seeds were moistened with distilled water (50 ml) and carefully shaken and drained.The germinated seeds were freeze-dried and milled. Both water and glass material were sterilized before use.

Figures 6.7 and 6.8 show the two cultivars of both chickpeas and lentils before and after germination.

FIGURE 6.7. Chickpeas Fardon and Blanco Lechoso before and after germination.

FIGURE 6.8. Lentils Magda 20 and Lyda before and after germination.

Table 6.5 shows the weight of the seeds before and after germination as well as the percentage of germination and final dry weight of the seeds.

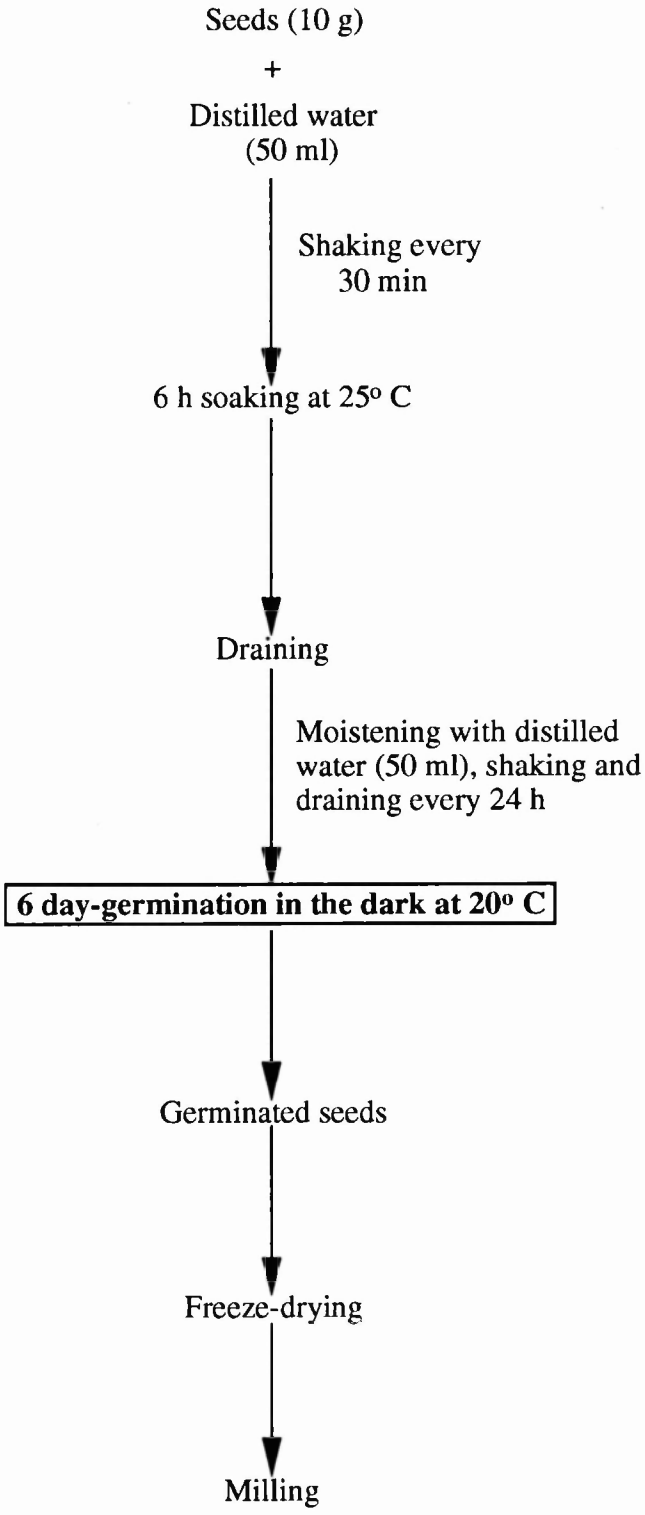
The germination process for both chickpeas and lentils has been summarized in Figure 6.9.

TABLE 6.5. Table of weights of seeds before and after germination, percentage of germination and dry weight of seeds for both chickpeas and lentils.

Cultivar and Treatment	*Before Germination*		*After Germination*			
	Weight seeds[a] (g)	*number seeds*	*Weight germinated seeds (g)*	*number germinated seeds*	*% gemination*	*Dry weight seeds[b] (g)*
Chickpeas Fardon						
unprocessed	10.0	32				9.2
germination	10.0	32	31.3	30	94	8.2
Chickpeas Blanco Lechoso						
unprocessed	10.0	19				9.1
germination	10.0	19	31.8	18	95	8.3
Lentils Magda 20						
unprocessed	10.0	112				9.5
germination	10.0	112	0.5	6	5	0.3
Lentils Lyda						
unprocessed	10.0	148				9.3
germination	10.0	148	34.3	138	93	8.4

[a] weight of the seeds after having been in a vacuum desiccator for 92 h.
[b] weight of both unprocessed and germinated seeds after freeze-drying.

FIGURE 6.9. Germination treatment for both chickpeas and lentils.



6.3.3 Saponin Analysis

The ground seeds were extracted with 70 % aqueous ethanol containing 0.01 % EDTA at room temperature. An internal standard of α-hederin was added to the sample prior to solvent extraction. The extracts were evaporated to dryness at less than 40°C and dissolved in a mixture of 2 ml of water and 1-butanol (1:1). After centrifugation, the 1-butanol layers were collected and analyzed by high-performance liquid chromatography.

Separations were performed on a column packed with Ultratechsphere 5 μ C_{18} and chromatographic runs were carried out with an acetonitrile-water gradient elution system. Solvents were acetonitrile-acetic acid (1000:0.3 v/v) (solvent A) and water:acetic acid:EDTA (1000:0.3:0.15 v/v/w) (solvent B). Solvents were degassed with helium. The gradient was run according to the following programme: 35% A isocratically for 20 min followed by an increase to 42% A after a further 4 min, held at 42% A for 10 min, then changed to 35% A over a further 4 min and finally held at 35% A for 6 min. The injection volume, flow rate and run time were 10 μl, 0.9 ml/min and 45 min, respectively, and detection was monitored by UV absorption at 205 nm as described in Section 5.3.2.

6.3.4 Statistical Analysis

The data were subjected to the multiple comparison method of Tukey using Minitab 8.21 Software (Macintosh version).

Tukey's method provides confidence intervals for all pairwise differences between level means. The null hypothesis of no difference between means is rejected if, and only if, zero is not contained in the confidence interval.

The family error rate in Tukey's method, which will be specified to be equal to 0.05, is the maximum probability of obtaining one or more confidence intervals that do not contain the true difference between level means.

6.3 RESULTS*

6.3.1 Soaking

6.3.1.1 *Hydration coefficient*

During the soaking process chickpeas and lentils absorb water which results in an increase in weight. The hydration coefficient is a measure of gross water uptake.

Table 6.6 shows the hydration coefficients of both chickpeas and lentils after having been soaked in distilled water, 0.1% citric acid and 0.07% sodium bicarbonate solutions for 24 h at 25°C.

The hydration coefficients for each cultivar of chickpeas or lentils were quite similar to each other regardless of the soaking solution (Table 6.6), although it seems that soaking in the citric acid solution brought about a smaller water uptake for all the studied seeds with the exception of lentils Magda 20, where this effect was not observed. This particular cultivar of lentils also showed the largest hydration coefficients of all the legume seeds studied in the three types of soaking media.

* The data in this chapter have been published in:

Ruiz, R.G., Price, K.R., Arthur, A.E., Rose, M.E., Rhodes, M.J.C. and Fenwick, G.R. (1996). *J. Agric. Food Chem.*, **44**: 1526-1530.

Ruiz, R.G., Price, K.R., Rose, M.E., Rhodes, M.J.C. and Fenwick, G.R. (1996). *Z. Lebensm. Unters. Forsch.*, **203** (in press).

TABLE 6.6. Hydration coefficients of each of the two cultivars of chickpeas and lentils after having been soaked in distilled water, 0.1 % citric acid and 0.07 % sodium bicarbonate solutions for 24 h at 25 °C.

Cultivar and treatment	*Hydration coefficient*
Chickpeas Fardon	
water soaking	218
citric acid soaking	209
s. bicarbonate soaking	215
Chickpeas Blanco Lechoso	
water soaking	227
citric acid soaking	226
s. bicarbonate soaking	229
Lentils Magda 20	
water soaking	235
citric acid soaking	232
s. bicarbonate soaking	230
Lentils Lyda	
water soaking	213
citric acid soaking	208
s. bicarbonate soaking	211

6.3.1.2 *Saponin content and composition*

The effect of soaking on the saponin content and composition of chickpeas and lentils is shown in Tables 6.7 and 6.8 and Figures 6.10 and 6.11.

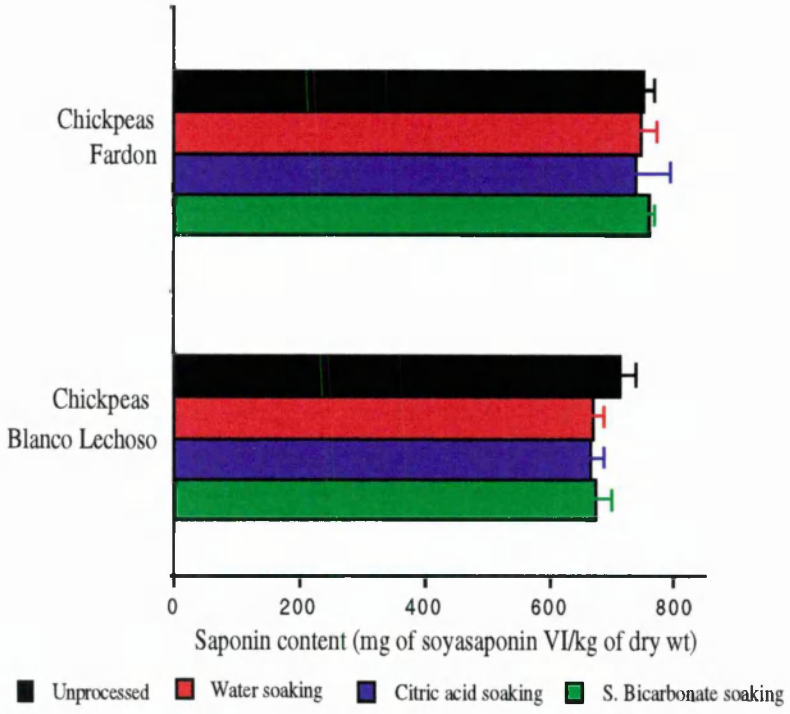


FIGURE 6.10. Saponin content of chickpeas before and after soaking treatments.

TABLE 6.7. Saponin content* of chickpeas before and after soaking treatments.

cultivar and treatment	*saponin content (mg of soyasaponin VI/ kg of dry wt)*	
	seeds	*soaking solution*
Chickpeas Fardon		
unprocessed	759 ± 14[a]	-----
24 h water soaking	744 ± 28[a]	ND
24 h citric acid soaking	737 ± 55[a]	ND
24 h s. bicarbonate soaking	758 ± 10[a]	ND
Chickpeas Blanco Lechoso		
unprocessed	711 ± 28[b]	-----
24 h water soaking	670 ± 19[b]	ND
24 h citric acid soaking	667 ± 21[b]	ND
24 h s. bicarbonate soaking	673 ± 25[b]	ND

*Values are the mean of four determinations ± standard deviation. The same superscripts in the same column indicate no significant differences (family error rate = 0.05).
ND, not detectable.

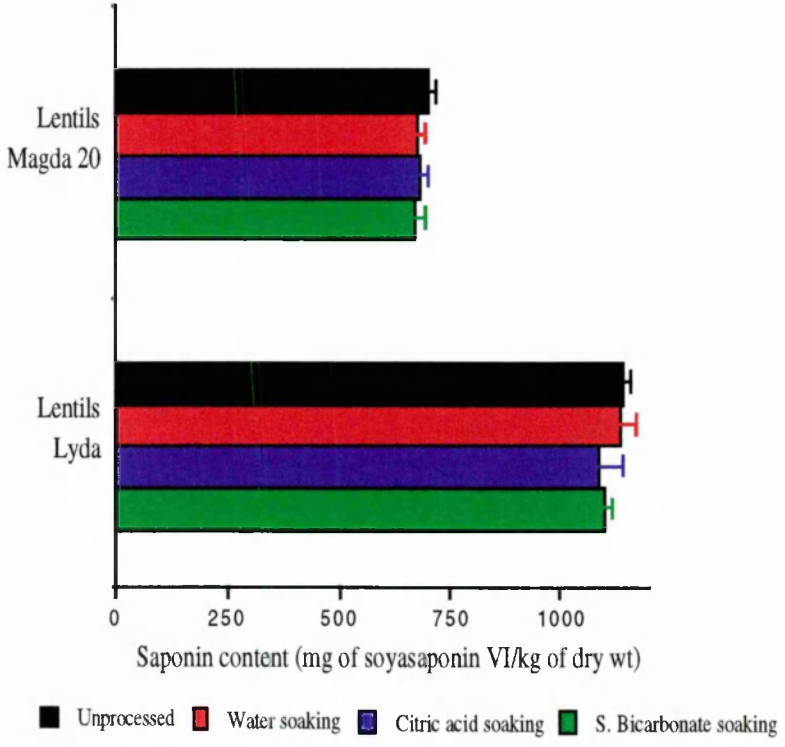


FIGURE 6.11. Saponin content of lentils before and after soaking treatments.

TABLE 6.8. Saponin content* of lentils before and after after soaking treatments.

cultivar and treatment	*saponin content (mg of soyasaponin VI/kg of dry wt)*	
	seeds	*soaking solution*
L. culinaris Magda 20		
unprocessed	703 ± 14[a]	-----
24 h water soaking	675 ± 18[a]	ND
24 h citric acid soaking	684 ± 18[a]	ND
24 h s. bicarbonate soaking	670 ± 26[a]	ND
L. culinaris Lyda		
unprocessed	1139 ± 15[b]	-----
24 h water soaking	1135 ± 36[b]	ND
24 h citric acid soaking	1088 ± 52[b]	ND
24 h s. bicarbonate soaking	1097 ± 20[b]	ND

*Values are the mean of four determinations ± standard deviation. The same superscripts in the same column indicate no significant differences (family error rate = 0.05).
ND, not detectable.

Soyasaponin VI (Figure 6.12) was the only saponin detected in both the unprocessed and soaked seeds of each of the two cultivars of chickpeas and lentils. No saponin was detected in any of the soaking solutions (Tables 6.7 and 6.8; Figures 6.10 and 6.11).

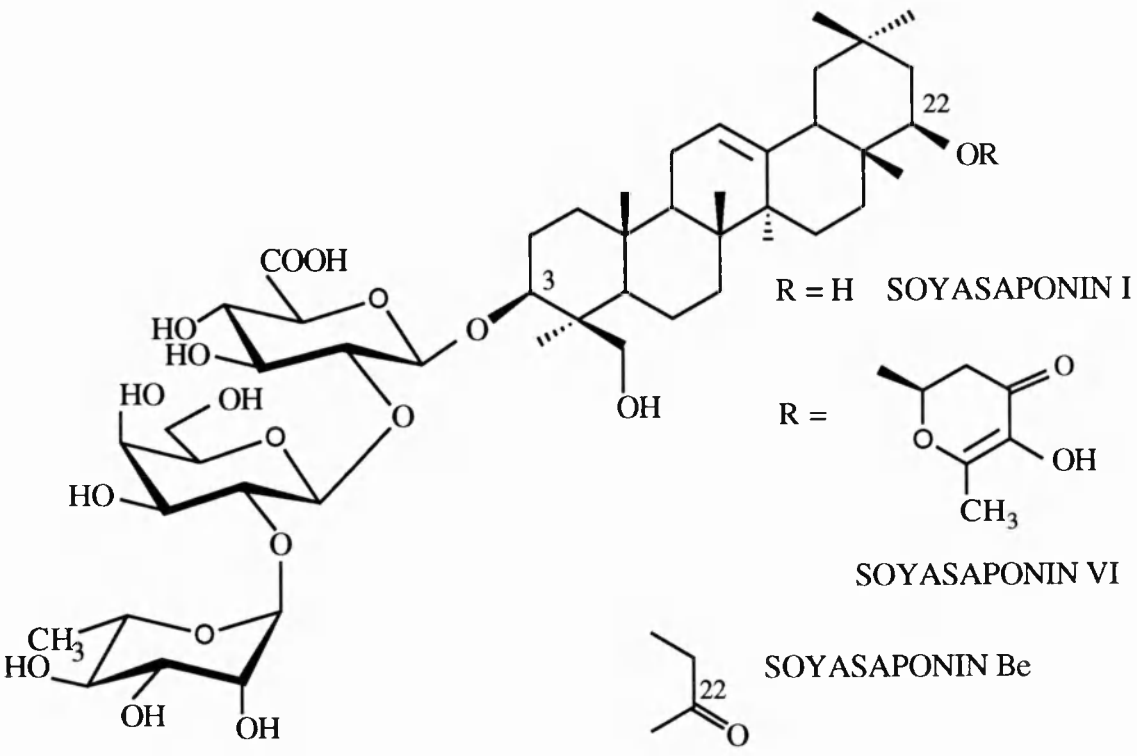


FIGURE 6.12. Chemical structures of soyasaponins I, VI and Be.

Figure 6.13 shows the chromatograms corresponding to the unprocessed seeds of chickpeas Fardon, chickpeas Blanco Lechoso, lentils Lyda and lentils Magda-20.

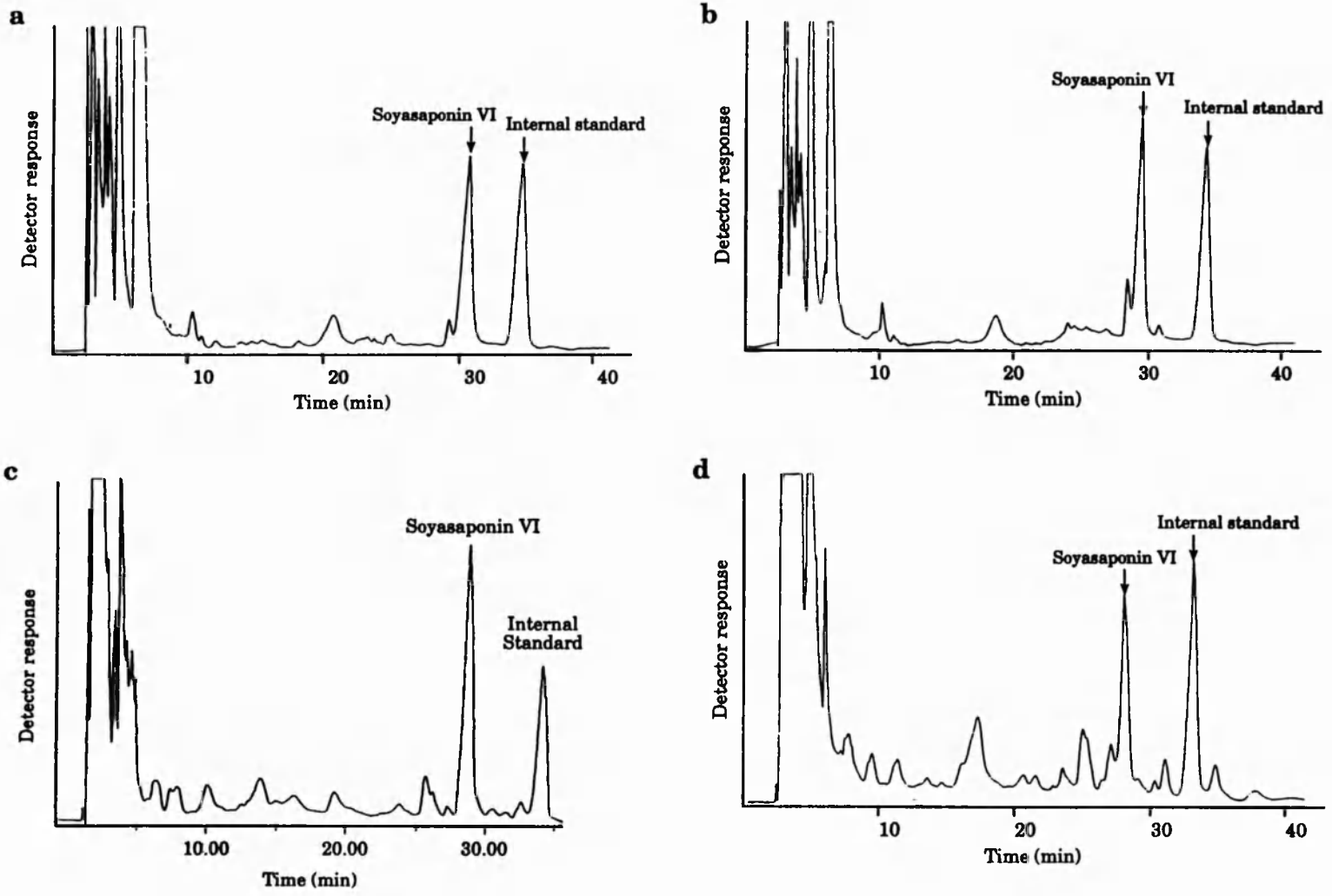


FIGURE 6.13. Chromatograms of saponins (soyasaponin VI) in unprocessed (a) chickpeas Fardon, (b) chickpeas Blanco Lechoso, (c) lentils Magda 20, and (d) lentils Lyda with internal standard (α-hederin).

No significant differences were found between the unprocessed and the soaked seeds. Therefore, neither the saponin content nor the composition of either chickpeas or lentils were affected by soaking regardless of the pH of the soaking solution (Tables 6.7 and 6.8; Figures 6.10 and 6.11).

6.3.2 Soaking plus cooking

6.3.2.1 *Hydration coefficient*

Table 6.9 shows the hydration coefficient of both chickpeas and lentils, which were soaked in distilled water for 12 h at 25°C prior to cooking.

TABLE 6.9. Hydration coefficients of each of the two cultivars of chickpeas and lentils after having been soaked in distilled water for 12 h at 25°C.

Cultivar	*Hydration coefficient*
Chickpeas Fardon	219
Chickpeas Blanco Lechoso	225
Lentils Magda 20	225
Lentils Lyda	212

The hydration coefficients of chickpeas Fardon, chickpeas Blanco Lechoso and lentils Lyda soaked in distilled water for 12 h (219, 225 and 212, respectively; Table 6.9) were very similar to the ones obtained after soaking these legumes in distilled water for 24 h: 218, 227 and 213, respectively (Table 6.6). Therefore, the maximum hydration

coefficients in the above mentioned legumes occurred at or before a time of 12 h soaking. However, this maximum water uptake had not been reached at 12 h soaking in lentils Magda 20 (hydration coefficient: 225; Table 6.9), which showed a larger hydration coefficient of 235 after 24 h (Table 6.6) than after 12 h soaking.

6.3.2.2 *Microscopic examination*

Figures 6.14, 6.15, 6.16 and 6.17 show the micrographs of unprocessed chickpeas Fardon, unprocessed lentils Lyda, 30 min-cooked chickpeas Fardon and 30 min-cooked lentils Lyda, respectively.

Tightly packed cells with sharp granules of starch, protein bodies and nucleus plus nucleolus are displayed in the microscope photographs corresponding to the unprocessed seeds (Figures 6.14 and 6.15).

The micrographs of the cooked seeds (Figures 6.16 and 6.17) exhibit a large separation of cells with swollen, misshapen, gelatinized granules of starch. However, starch swelling is limited by cell walls, which remained intact.

The seed structure in the case of 30 min-cooked lentils was more disrupted (Figure 6.17) than that corresponding to chickpeas cooked for the same length of time because a greater separation of cells can be observed for the former (Figure 6.17).

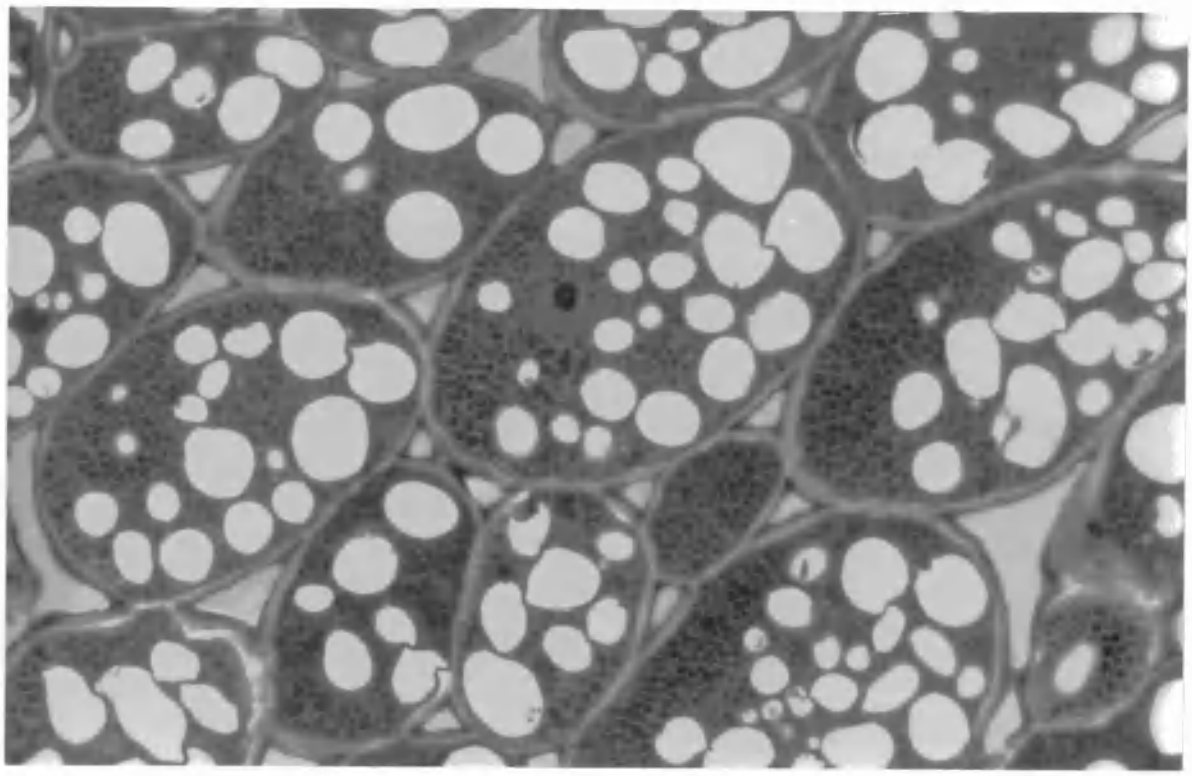


FIGURE 6.14. Microscope photograph (x 480) of unprocessed chickpeas Fardon.

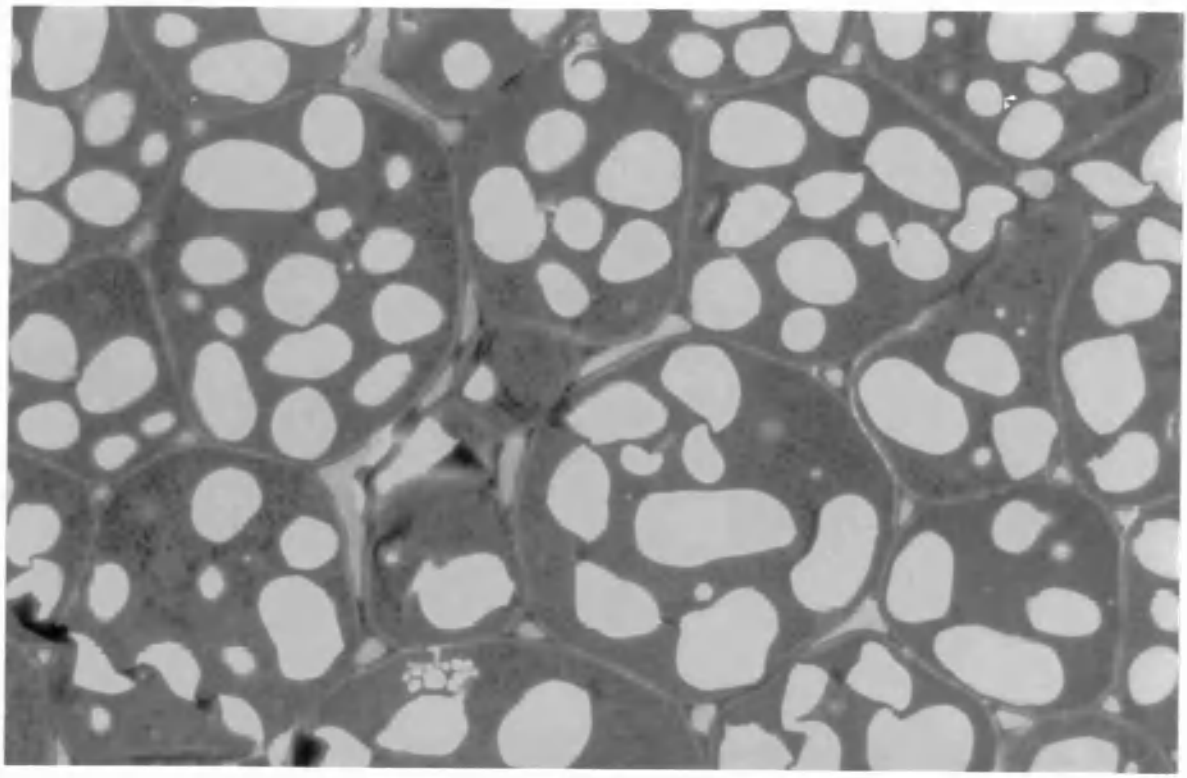


FIGURE 6.15. Microscope photograph (x 480) of unprocessed lentils Lyda.

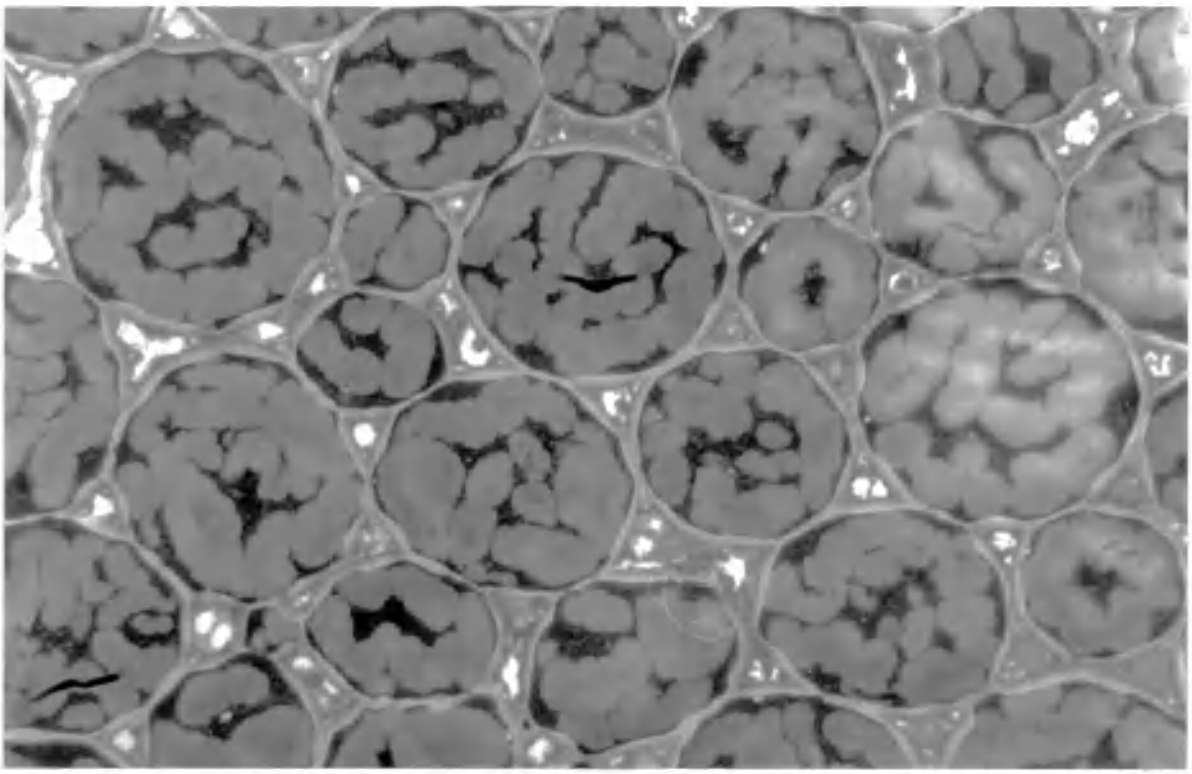


FIGURE 6.16. Microscope photograph (x 480) of 30 min-cooked chickpeas Fardon.

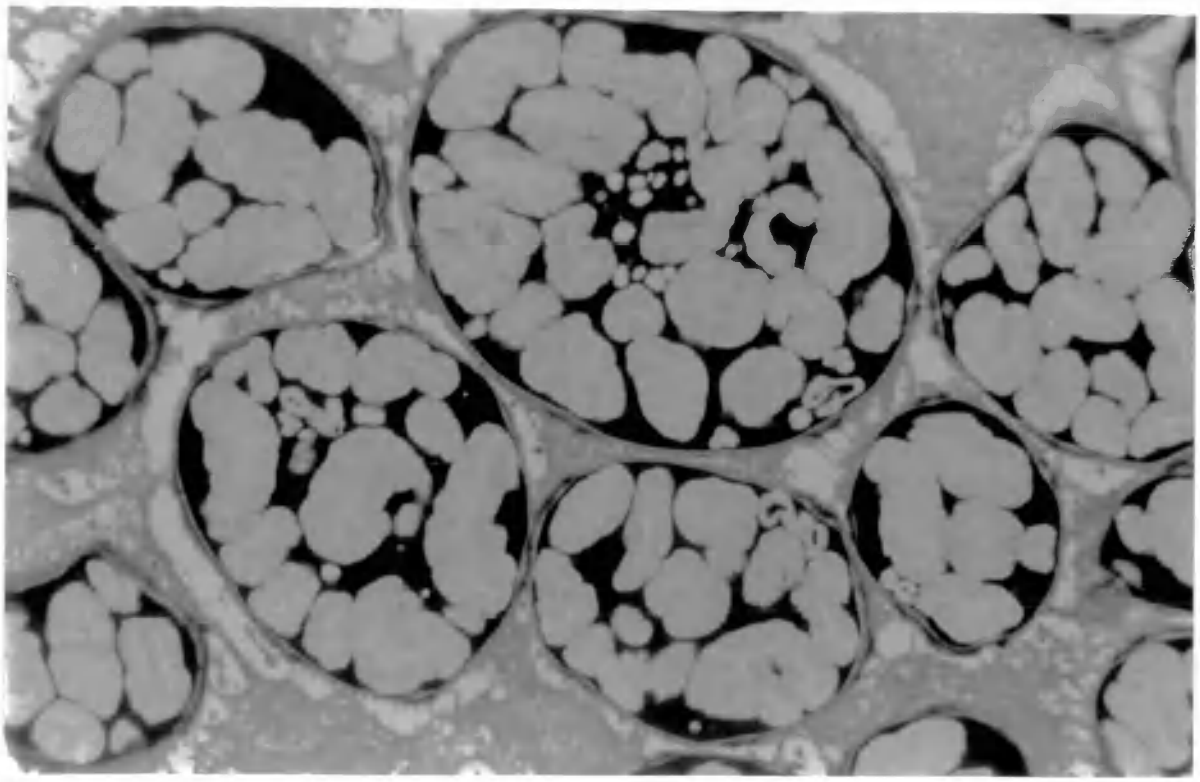


FIGURE 6.17. Microscope photograph (x 480) of 30 min-cooked lentils Lyda.

6.3.2.3 *Saponin content and composition*

The effect of cooking on the saponin content and composition of chickpeas and lentils is shown in Tables 6.10 and 6.11 and Figures 6.18 and 6.19.

Both soyasaponins VI and I (Figure 6.12) were detected in all the cooked seeds of both chickpeas and lentils. Soyasaponins VI and I were also detected in all the cooking solutions from both cultivars of lentils (Tables 6.10 and 6.11; Figures 6.18 and 6.19). However, soyasaponin VI was only detected in the cooking solutions from both cultivars of chickpeas after they had been cooked for 30 min but not in the ones cooked for 60, 90 or 120 min (Table 6.10; Figure 6.18).

Leaching of saponins into the cooking solutions was observed, which ranged from 2 to 4%, 3 to 5%, 12 to 14% and 6 to 7% for chickpeas Fardon, chickpeas Blanco Lechoso, lentils Magda-20 and lentils Lyda respectively (Tables 6.10 and 6.11; Figures 6.18 and 6.19). The total saponin content of the cooking solutions from both cultivars of chickpeas showed a steady increase with cooking time, whereas leaching of saponins in the case of lentils appeared to cease after 30 min cooking (Tables 6.10 and 6.11; Figures 6.18 and 6.19).

Conversion of soyasaponin VI into soyasaponin I was also observed in all the seeds studied (Tables 6.10 and 6.11; Figures 6.18 and 6.19), which increased with cooking time; as soyasaponin VI decreased, soyasaponin I increased for both seeds and cooking solutions. Figure 6.20 shows the chromatograms corresponding to saponins present in chickpeas Fardon cooked for 30-120 min.

While the total saponin contents of both chickpeas Fardon and Blanco Lechoso cooked for 30-120 min (Table 6.10; Figure 6.18), plus those of their corresponding cooking solutions, do not differ statistically from the total saponin content of the unprocessed seeds, the total saponin contents of both cultivars of lentils are significantly lower than those of the raw seeds (Table 6.11; Figure 6.19); the saponin losses were 27 to 31% for the former and 15 to 19% for the latter.

TABLE 6.10. Saponin content* of chickpeas before and after soaking plus cooking treatment.

cultivar and treatment	saponin content (mg/kg of dry wt)							
	seeds			cooking solution				total saponin content of seeds and solutions
	soyasaponin I	soyasaponin VI	total	soyasaponin I	soyasaponin VI	total	% saponin leaching	
Chickpeas Fardon								
• unprocessed	ND	759 ± 14	759 ± 14					759 ± 14[a]
• 12 h water soaking plus 30 min of cooking in water	407 ± 17	339 ± 13	746 ± 24	10 ± 0	5 ± 0	15 ± 1	2	761 ± 24[a]
• 12 h water soaking plus 60 min of cooking in water	513 ± 18	227 ± 21	740 ± 30	18 ± 2	ND	18 ± 2	2	758 ± 30[a]
• 12 h water soaking plus 90 min of cooking in water	585 ± 15	141 ± 4	726 ± 13	23 ± 2	ND	23 ± 2	3	749 ± 13[a]
• 12 h water soaking plus 120 min of cooking in water	640 ± 8	83 ± 7	723 ± 7	30 ± 2	ND	30 ± 2	4	753 ± 10[a]
Chickpeas Blanco Lechoso								
• unprocessed	ND	711 ± 28	711 ± 28					711 ± 28[b]
• 12 h water soaking plus 30 min of cooking in water	408 ± 20	274 ± 6	682 ± 17	13 ± 1	5± 0	18 ± 1	3	700 ± 17[b]
• 12 h water soaking plus 60 min of cooking in water	519 ± 15	142 ± 15	661 ± 28	25 ± 2	ND	25 ± 2	4	686 ± 26[b]
• 12 h water soaking plus 90 min of cooking in water	543 ± 20	120 ± 7	663 ± 25	27 ± 3	ND	27 ± 3	4	690 ± 24[b]
• 12 h water soaking plus 120 min of cooking in water	588 ± 18	88 ± 2	676 ± 19	36 ± 6	ND	36 ± 6	5	712 ± 17[b]

* Values are the mean of four determinations ± standard deviation. The same superscripts in the same column indicate no significant differences (family error rate = 0.05). ND, not detectable.

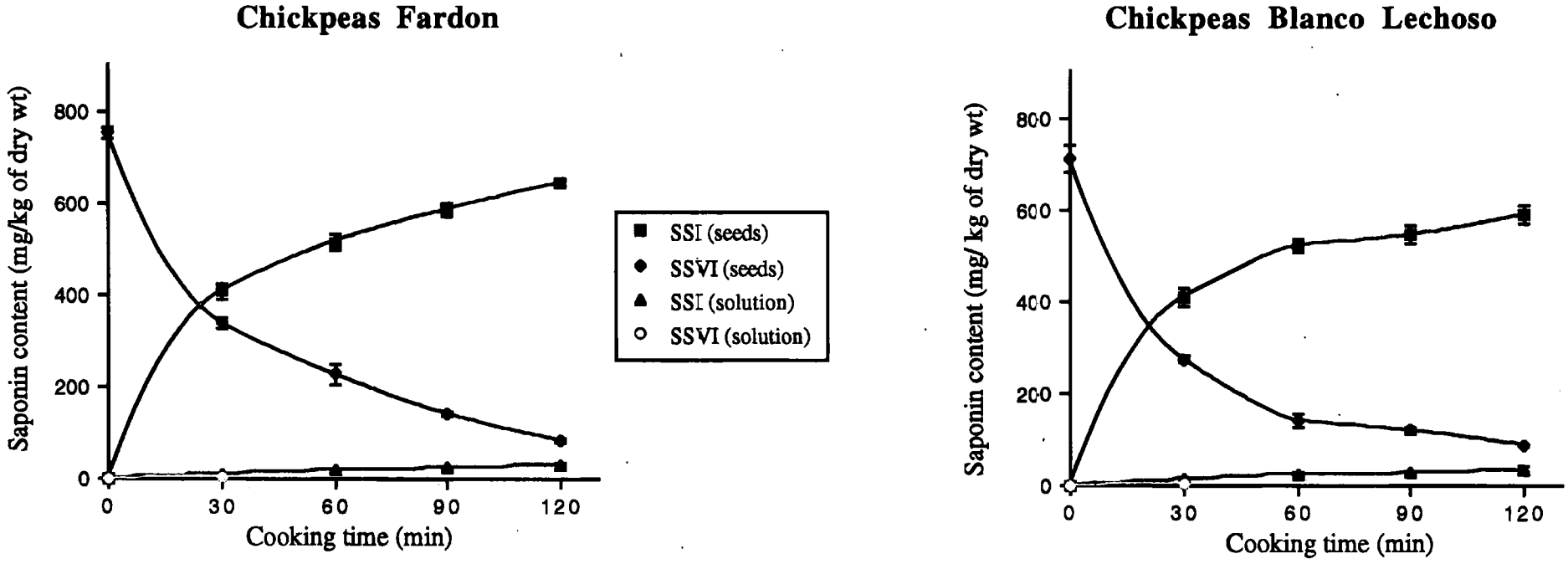


FIGURE 6.18. Changes in saponin content (mg/kg of dry wt) of chickpeas after cooking.

TABLE 6.11. Saponin content* of lentils before and after soaking plus cooking treatment.

cultivar and treatment	saponin content (mg/kg of dry wt)							
	seeds			cooking solution				
	soyasaponin I	soyasaponin VI	total	soyasaponin I	soyasaponin VI	total	% saponin leaching	total saponin content of seeds and solutions
Lentils Magda 20								
• unprocessed	ND	703 ± 14	759 ± 14					703 ± 14[a]
• 12 h water soaking plus 30 min of cooking in water	105 ± 11	319 ± 9	424 ± 10	27 ± 0	40 ± 6	67 ± 4	14	491 ± 13[b]
• 12 h water soaking plus 60 min of cooking in water	176 ± 14	276 ± 7	452 ± 12	35 ± 1	29 ± 1	64 ± 0	12	516 ± 12[b]
• 12 h water soaking plus 90 min of cooking in water	191 ± 10	228 ± 19	419 ± 29	44 ± 1	25 ± 1	69 ± 0	14	488 ± 30[b]
• 12 h water soaking plus 120 min of cooking in water	233 ± 11	200 ± 13	433 ± 12	48 ± 0	22 ± 0	70 ± 0	14	503 ± 12[b]
Lentils Lyda								
• unprocessed	ND	1139 ± 15	1139 ± 15					1139 ± 15[c]
• 12 h water soaking plus 30 min of cooking in water	381 ± 21	526 ± 36	907 ± 56	35 ± 1	20 ± 0	55 ± 1	6	962 ± 56[d]
• 12 h water soaking plus 60 min of cooking in water	510 ± 20	393 ± 43	903 ± 43	41 ± 4	12 ± 0	53 ± 3	6	956 ± 41[d]
• 12 h water soaking plus 90 min of cooking in water	519 ± 21	342 ± 11	861 ± 33	49 ± 1	10 ± 1	59 ± 1	6	920 ± 32[d]
• 12 h water soaking plus 120 min of cooking in water	543 ± 9	330 ± 26	873 ± 18	60 ± 0	6 ± 0	66 ± 0	7	939 ± 18[d]

* Values are the mean of four determinations ± standard deviation. The same superscripts in the same column indicate no significant differences (family error rate = 0.05). ND, not detectable.

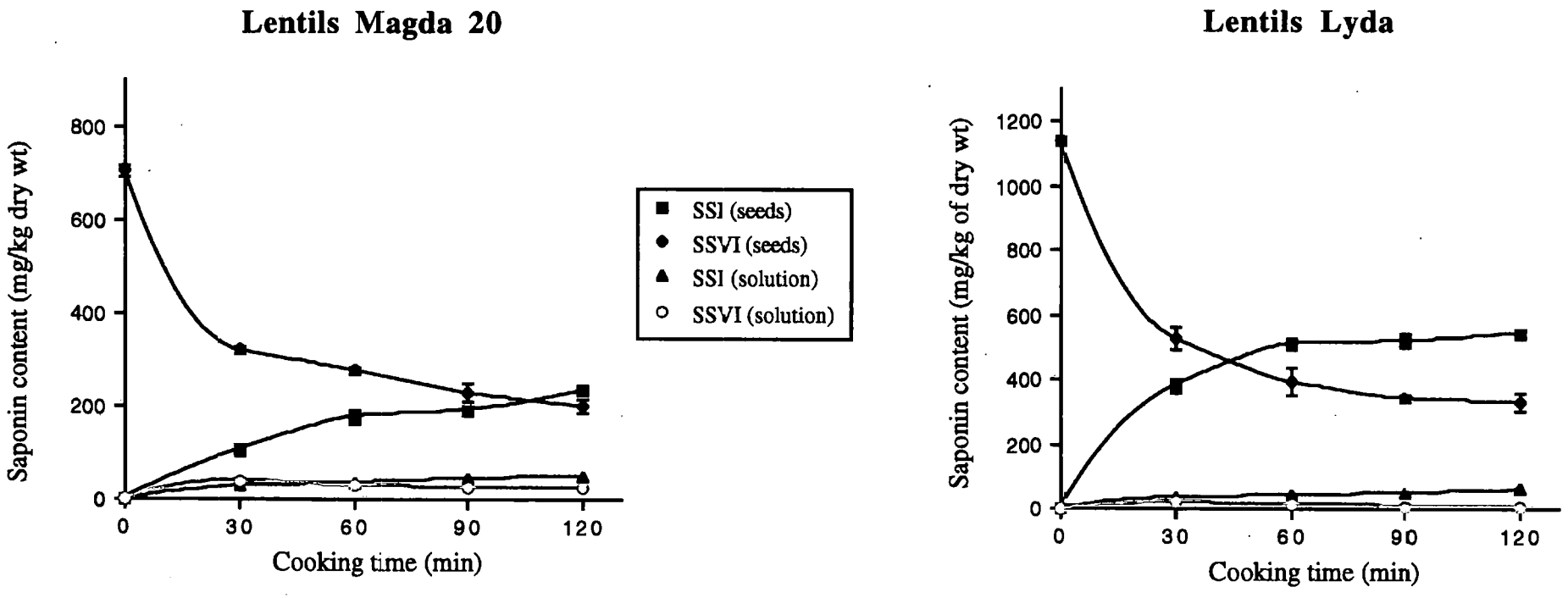


FIGURE 6.19. Changes in saponin content (mg/kg of dry wt) of lentils after cooking.

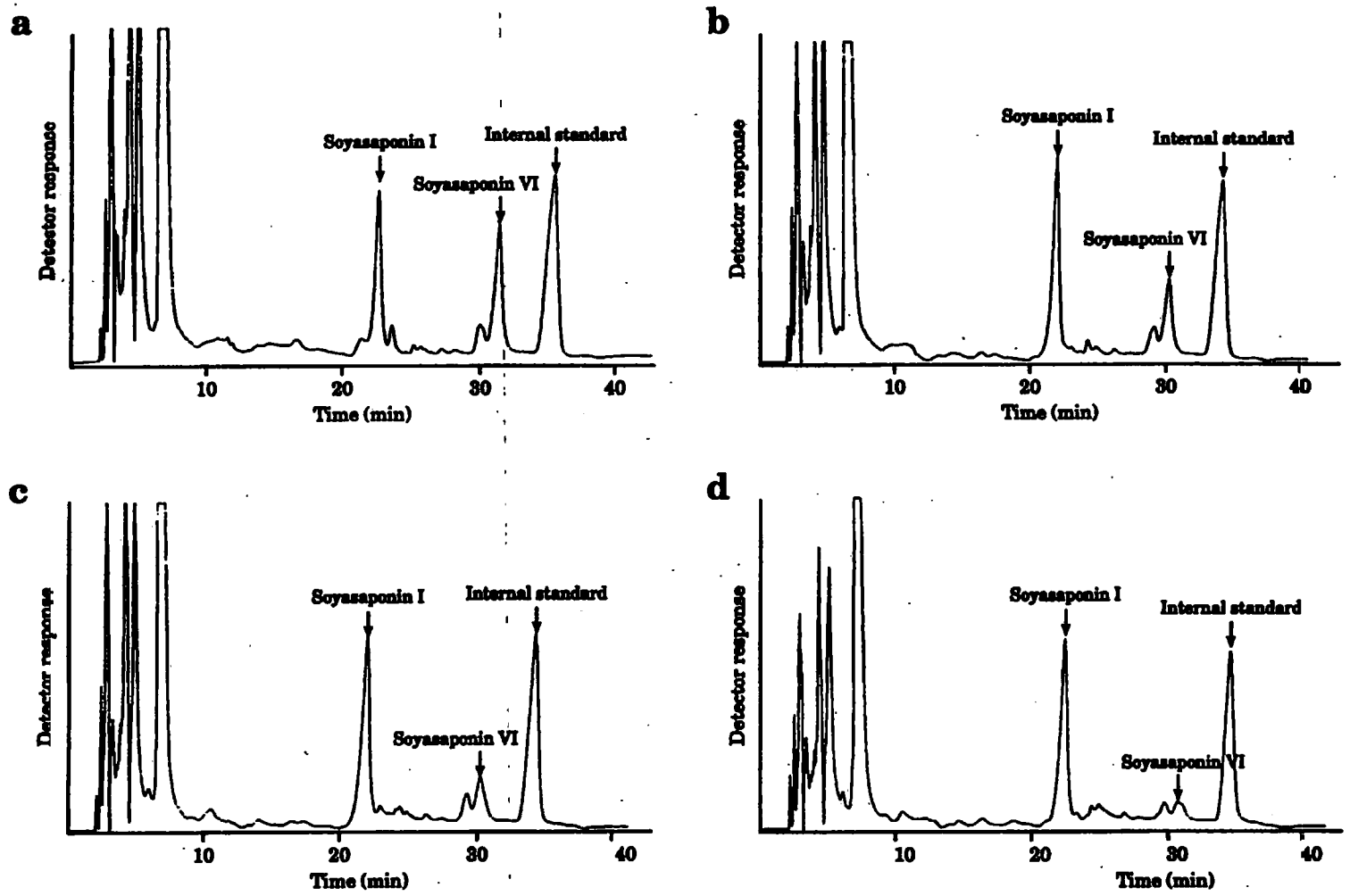


FIGURE 6.20. Chromatograms of saponins (soyasaponins VI and I) in chickpeas Fardon after cooking for (a) 30, (b) 60, (c) 90, and (d) 120 min with internal standard (α-hederin).

6.3.3 Germination

The percentage of germination for chickpeas Fardon, chickpeas Blanco Lechoso, lentils Magda-20 and lentils Lyda was 94, 95, 5 and 93% respectively (see Table 6.5). Since an acceptable percentage of germination was not achieved for lentils Magda 20, analysis of saponins in this particular cultivar of lentils was not carried out.

The effect of germination on the saponin content and composition of chickpeas Fardon, chickpeas Blanco Lechoso and lentils Lyda is shown in Table 6.12 and Figure 6.21.

Soyasaponin VI (Figure 6.12) was the only saponin detected in both the unprocessed and germinated seeds of the two cultivars of chickpeas (Fardon and Blanco Lechoso) and the cultivar of lentils (Lyda).

No significant differences between unprocessed and germinated seeds were found for both total saponin content or for changes in composition (Table 6.12 and Figure 6.21).

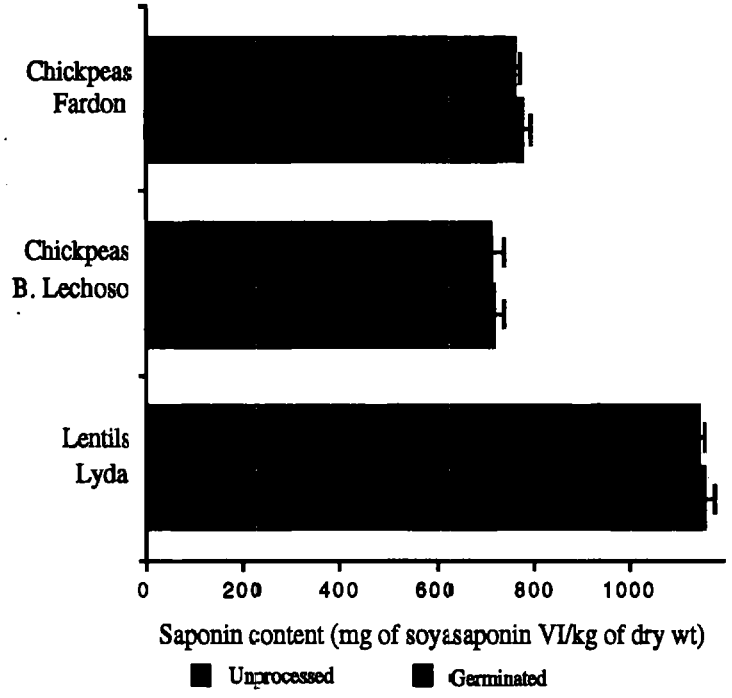


FIGURE 6.21. Saponin content of chickpeas and lentils before and after germination treatment.

TABLE 6.5. Saponin content (on a dry matter basis)* of chickpeas and lentils before and after germination treatment.

	saponin content	
cultivar and treatment	*mg/kg*	*µg/seed*
Chickpeas Fardon		
unprocessed	759 ± 14[a]	216 ± 4[a]
germination	777 ± 17[a]	212 ± 5[a]
Chickpeas Blanco Lechoso		
unprocessed	711 ± 28[b]	340 ± 14[b]
germination	717 ± 22[b]	330 ± 10[b]
Lentils Lyda		
unprocessed	1139 ± 15[c]	72 ± 1[c]
germination	1155 ± 21[c]	70 ± 1[c]

*Values are the mean of four determinations ± standard deviation. The same superscripts in the same column indicate no significant differences (family error rate = 0.05).

6.4 DISCUSSION

6.4.1 Soaking and cooking

6.4.1.1 *Hydration coefficient*

The results illustrated in Table 6.6 for both lentils Magda 20 and lentils Lyda are in good agreement with Abou-Samaha *et al.*[289], who found that two different varieties of lentils soaked in water weighed slightly more than those soaked in sodium bicarbonate solution solution. According to these authors the rate of increase of hydration coefficient was high in the first hours of soaking, and then decreased with extension of steeping time. The maximum value for this parameter was obtained after 12 and 9 h of soaking in water and saline solution, respectively, in both varieties of lentils.

A rapid uptake of water is a desirable attribute of legume grain used for food. The rate of penetration of water is affected by seed size, seed hardness and permeability of the seed coat to water[290]. According to Bhatty[291] a good-cooking lentil has a short cooking time, which is partly a cultivar characteristic, uniformity of the cooked lentils, ability of the hull to stay attached to the seed during the cooking process, and acceptable taste, flavour and appearance of both the raw and cooked lentils. This author[291] tried to correlate the cooking quality of a Canadian cultivar of lentils with water uptake rate. Although the poor-cooking lentils had smaller hydration coefficients than the good-cooking lentils, both the good- and poor-cooking lentils absorbed water at about the same rate, suggesting that smaller water uptake was not responsible for the poor-cooking condition. However, the maximum hydration coefficient in the good-cooking lentils occurred at about 12 h whereas this maximum in the poor-cooking lentils was not reached until 24 h soaking even though the highest rate of increase of the hydration coefficient in both types of lentils took place in the first hours of soaking. This situation seems to be analogous to that of lentils Lyda and lentils Magda 20. While the former had similar hydration coefficients after 12 and 24 h soaking, the water uptake of the latter did not

stop after 12 h soaking (Tables 6.6 and 6.9). This together with the fact that lentils Magda 20 lacked almost all the quality attributes for a good-cooking lentil mentioned above, might suggest that this particular cultivar of lentils fits the poor-cooking conditions.

Khan *et al.*[292] found that the water uptake of *desi* chickpeas was lower than that of *kabuli* type. This finding is in accordance with the results in Tables 6.6 and 6.9, which show chickpeas Blanco Lechoso (type *kabuli*) to have higher hydration coefficients than chickpeas Fardon (type *desi*). According to Altia *et al.*[293], the rates of hydration of two cultivars of *kabuli* chickpeas were high during the first 4 h of soaking and approached zero after 12 h.

6.4.1.2. *Saponin content and composition*

Soaking did not modify the saponin content or composition of chickpeas and lentils regardless of the pH of the soaking solution. Soyasaponin VI, within the seed matrix, was stable under the soaking conditions described above, although, in contrast, DDMP saponins have been reported to be stable at acidic pH and in the presence of hydrogen peroxide[262] but unstable in basic pH[262] and in the presence of ferric ion[258].

During cooking, the native saponin, soyasaponin VI, was partially degraded into soyasaponin I, and both of these saponins leached into the cooking solution. Leaching of saponins into the cooking solutions seemed to be more important in lentils (6-14%), particularly for lentils Magda-20 (12-14%), than in chickpeas (2-5%), which can be attributed to a larger disruption of the seed structure in the case of lentils. This can be confirmed with the observation of micrographs shown in Figures 6.16 and 6.17 where the separation of cells, which is indicative of the alteration of the seed structure as a consequence of the heat treament, is greater for lentils.

An overall loss of saponin content was found for lentil (15-31% loss), but none was observed for chickpea.The saponin losses in the case of lentils might be attributed to a possible conversion of soyasaponin VI into soyasaponin I plus soyasaponin Be (Figure

6.12), which possesses soyasapogenol E (Figure 1.6) as aglycone and the same sugar chain as soyasaponin I. Okubo *et al.*[258] found that soyasaponin VI easily changed into soyasaponin I and Be in the presence of Fe^{3+}. However, since the cooking experiment was carried out using a teflon-coated pan, the presence of Fe^{3+} would be unlikely. Therefore, the reduction in saponin level reported for lentils is more likely to be due to the fact that, apart from conversion of soyasaponin VI into soyasaponin I, the latter might suffer further degradation as a consequence of heating, losing the different sugars of its glycosidic chain.

The differences in saponin recovery between chickpeas and lentils found in the present study could be attributed to the fact that in the case of lentils, especially the cultivar Magda-20, the seed structure was highly disrupted, releasing more saponins into the cooking solution. The saponins might be then more liable to suffer damage by heat whereas for the chickpeas, whose structure was modified to a much smaller extent by heating, soyasaponin VI and I would be more protected and suffer less degradation in the protective environment supplied by the seed.

Studies on the effect of soaking and cooking on saponin content and composition are very few and contradictory. While Fenwick and Oakenfull[126] found that cooking had little effect on broad beans, Khokhar and Chauan[151] reported that soaking in a solution of mixed mineral salt removed much more of the saponins in moth beans than did soaking in water (30 to 36% and 9 to 18% respectively) and ordinary cooking of the seeds presoaked in water reduced saponin level by 12-15%. Jood *et al.*[152] also found that common domestic processing and cooking treatments reduced the saponin level of chickpeas and black grams significantly. However, since the assay[116] used in both cases probably did not distinguish between saponins and oligosaccharides, and as the conditions of soaking have been found to have a significant effect on the oligosaccharide contents of legumes[280], these results should be treated with caution.

It has already been mentioned that some of the biological properties reported for saponins are now recognised to be dependent on their particular chemical structures

(Sections 5.1 and 6.1). In this sense, Yoshiki *et al.*[266] have found DDMP saponins to scavenge oxygen radicals (see Section 1.2.10.4) and the scavenging activity related to the DDMP moiety attached to the triterpene aglycone. In the organism, oxygen radicals have been implicated in the initiation and development of various diseases, often mediated through chain reactions of lipid peroxidation and DNA- and protein-binding and degeneration[267,268]. The present study has shown that important physiological activities, ascribed to DDMP saponins in relation to the prevention of biomolecular damage due to radical attack, might be partial or totally lost as a consequence of certain types of legume processing such as cooking.

6.4.2 Germination

Studies on the effect of germination on saponin content and composition are also scant and inconsistent. Whilst saponins were found in both sprouted alfalfa[126], mungbean[126] and *M. lupulina*[164], they were reduced in quantity when moth bean[151], chickpea[151], blackgram[153], rice bean[154], faba bean[155] and pea[156] were sprouted. Nevertheless, the method of analysis of saponins[116] in these germination studies[151, 153-156] was the same as that used in the few experiments which have been carried out until now on the effect of cooking on saponin content[151,152]. The lack of reliability of this method has already been discussed in Section 6.4.1.2.

Conversion of soyasaponin VI into I was observed in both chickpeas and lentils as a consequence of cooking (see Section 6.3.2). However, only soyasaponin VI was detected in the germinated chickpeas and lentils. This finding is in good agreement with Tsurumi *et al.*[253], who found chromosaponin I (also called soyasaponin VI or βg; see Section 1.2.10.1) in all parts of seven-day-old etiolated pea seedlings but at higher concentration in the hook and root tip than in other non-growing tissues. However, soyasaponin I was reported to be almost undetectable. Although these authors gave

detailed information on the distribution of chromosaponin I in seven-day-old pea seedlings, no data were provided about the saponin content of the unprocessed peas. Therefore, no information about the effect of etiolation in pea could be obtained to compare with results given here.

Shimoyamada and Okubo[157] observed that germination in the dark of soyabean seed changed the composition of soyasaponins in the sprout. Germination under light caused a slight increase in the amount of soyasaponin I in the cotyledon, while in the sprout, the amounts of soyasaponins I, II and V (see Figure 1.5), especially I, increased greatly. These authors observed soyasaponins I-V in unprocessed and germinated soyabeans instead of their DDMP conjugated forms which is probably due to the harsher conditions they used for the extraction of saponins (hot solvent extraction at 80°C). However, as discussed earlier, DDMP-conjugated saponins have been proved to be the genuine saponins in the case of native soyabeans seeds[254,256] whilst soyasaponins I-V, the saponins normally associated with soya following conventional exhaustive hot solvent extraction, were shown to be artifacts derived from degradation of the DDMP saponins during the extraction process (see Section 1.2.10). Results from Section 5.4.2 have also confirmed this finding in the case of soyasaponin VI being the native saponin in lupin seed. On the other hand, soyasaponin VI has also been shown to be the only saponin present in both unprocessed chickpeas and lentils (Sections 6.3.1.2, 6.3.2.3 and 6.3.3) while soyasaponin I was a degradation product of soyasaponin VI as a consequence of heating.

Since the germination experiment described here was planned using normal commercial conditions, with germinated seed not being divided between cotyledon and sprout and using dark conditions (according to the way soyabean sprouts are produced in the Far East[283]), results were difficult to compare with those obtained by Shimoyamada and Okubo[157]. Nevertheless, as the major changes in saponin content during germination of soyabeans occurred in the sprout and the results in Table 6.12 and Figure 6.21 did not show significant changes in overall saponin content, this would suggest that saponins

may be mobilized from the cotyledon to the sprout during the germination process of chickpeas and lentils.

It is known that chickpea and lentil are legumes with relatively low saponin content when compared to soyabean, haricot or kidney bean[50]. Since germination did not modify their saponin content, these two legumes can, from a nutritional point of view, be consumed in a germinated form without the risk of deleterious properties, such as bitterness or astringency (see Section 1.2.9.3) ascribed to group B saponins[193], being developed as a consequence of germination. On the other hand, the beneficial radical scavenging properties attributed to soyasaponin VI do not disappear as a consequence of germination since no conversion of soyasaponin VI into I is produced and is in contrast to that which occurred when chickpeas or lentils are cooked.

6.5 CONCLUSIONS

- The work described here provides quantitative results, using intact saponins rather than their products from acid hydrolysis, made for the first time on the effect of soaking, cooking and germination of chickpeas and lentils, not only on the total saponin content but also on the true saponin composition.

- These data have established the fate of the native saponin, soyasaponin VI, in the seed matrix. The soyasaponin has been shown to be stable after soaking, regardless of the pH of the soaking solution, and germination but unstable when chickpeas and lentils were cooked.

- Soyasaponin VI was partially degraded during cooking into soyasaponin I, and both of these saponins leached into the cooking solution, 2-5% and 6-14% for chickpea and lentil, respectively. An overall loss of saponin content was found for lentil (15-31%

loss), but none was observed for chickpea.

- Therefore, this study proves that the important physiological properties ascribed to DDMP saponins are dependent upon the type of processing used on these legumes.

VII General Conclusions and Future Possibilities

7.1 CONCLUSIONS

7.1.1 Effect of cultivar and environment on saponin content of Australian sweet lupin seed

The saponin content of lupin seed from 5 cultivars of sweet *Lupinus angustifolius* and one cultivar of sweet *L. albus*, grown over 2 years in 4 regions of Australia with up to 15 plots sampled within each region, was estimated using a GC method, which quantifies the sapogenol resulting from the acid hydrolysis of the saponins, and ranged from 379 to 740 mg kg^{-1} in the harvested *L. angustifolius* seed whereas no saponin was detected in *L. albus*.

Three saponins were detected in the *L. angustifolius* grown in Australia, two of which possessed chromatographic properties identical to soyasaponin I and soyasaponin VI, which is a DDMP-conjugated form of soyasaponin I, while the third, at a much lower concentration, appeared to possess a novel structure.

The changes observed in saponin content of Australian *L. angustifolius* were found to be mainly dependent on cultivar, followed by the site and then the region where grown.

No significant effect was found between growing seasons although there was a strong correlation between the saponin content of Australian *L. angustifolius* and the site grown. This suggests that the level of saponin in the lupin seed could be highly influenced by specific environmental factors at a particular plot.

7.1.2 Effect of seed character on saponin content of lentil seed

The saponin content of seed from 20 cultivars of lentils (*Lens culinaris* Medik.) with

different seed sizes and testa colours, grown over 5 years in 18 provinces of Spain, was also determined by GC and ranged from 654 to 1269 mg kg^{-1}.

Two saponins were detected and identified as soyasaponins I and VI in all of the 20 cultivars of Spanish lentils.

Changes observed in the saponin content were found to be dependent on both seed size and testa colour. The total saponin content of *microsperma* lentils was significantly lower (929 mg kg^{-1}) than that of *macrosperma* seeds (1105 mg kg^{-1}). Lentils with a beige or green testa were found to have significantly higher saponin contents (1106 and 1089 mg kg^{-1}, respectively) than those with a brown testa (800 mg kg^{-1}).

7.1.3 Isolation of a novel saponin

A new saponin (~ 25 mg) was isolated from Australian *Lupinus angustifolius* using a combination of reversed-phase silica (C_{18}) flash chromatography with aqueous methanol and normal phase silica column chromatography with chloroform:methanol:water mixtures.

Both FAB and ES mass spectra allowed the relative molecular mass of the novel saponin to be deduced as 1104, and soyasapogenol B was confirmed to be its aglycone by GC-MS.

Although both reversed- and normal phase TLC plates displayed distinct single spots indicating a high degree of organic purity, very weak signals in the NMR spectrum were obtained, which made it impossible to elucidate the chemical structure of the new saponin. Nevertheless, from its mass spectra and chromatographic properties, it is postulated to be a new *O*-22 hexose conjugate of soyasaponin I.

7.1.4 Determination of saponins in lupin seed using high-performance liquid chromatography

A quantitative method for the determination of intact saponins in *Lupinus angustifolius* seed by HPLC was developed. The seed was extracted under mild conditions with 70% aqueous ethanol containing 0.01% EDTA and quantified using an internal standard of α-hederin. The saponin content of the *L. angustifolius* seed using this new method was estimated to be 450 mg kg^{-1}. This value is a 14% higher than that obtained from the conventional GC method, which suggests that saponin losses occurred during some of the stages involved in the latter method.

Soyasaponin VI was the only saponin detected in the lupin seed. Therefore, soyasaponin I is an artifact derived from degradation of soyasaponin VI during the extraction or concentration processes included in the GC approach.

The new technique was also shown to have several advantages over two other HPLC methods: namely, one for the analysis of derivatized saponins, which suffered from stability and selectivity problems, and another for the qualitative determination of intact saponins.

7.1.5 Effect of processing on the saponin content and composition of chickpea and lentil seed

The new HPLC method allowed the investigation of changes not only in the total saponin content but also in the true saponin composition of two cultivars of both chickpeas and lentils after the seeds were soaked in distilled water, citric acid, and sodium bicarbonate solutions, germinated in the dark for 6 days at 20°C, and cooked for 30, 60, 90, and 120 min after the seeds were presoaked in distilled water.

Neither soaking, regardless of the pH of the soaking solution, nor germination modifies the saponin content or composition of chickpeas and lentils.

The native saponin, soyasaponin VI, was partially degraded during cooking into soyasaponin I, and both of these saponins leached into the cooking solution to the extent of 2-5% and 6-14% for chickpea and lentil, respectively. An overall loss of saponin content was found for lentil (15-31% loss), but none was observed for chickpea. This may be attributed to the fact that in the case of lentils the seed structure was highly disrupted, releasing more saponins into the cooking solution. The saponins might then be more liable to suffer damage without the protective environment supplied by the seed.

This investigation has shown that the important physiological properties ascribed to DDMP saponins in relation to the prevention of biomolecular damage due to radical attack might be partial or totally lost as a consequence of legume cooking. However, their action would be complemented by the formation of maltol, which is also a good free radical scavenger.

7.2 FUTURE POSSIBILITIES

The results in Chapter II showed a strong correlation between the saponin content of the lupin (*Lupinus angustifolius*) seeds and the site grown. Therefore, it can be inferred that specific environmental factors at a particular plot could be responsible for high levels of saponins in the lupin seeds. A further study of such factors at a specific site such as temperature, rainfall, soil pH and fertilisers would be needed to decide which of these are important to grow lupin plants producing high or low saponin-containing seeds.

From the results in Chapter III it can be deduced that *macrosperma* lentils with beige or green testa are likely to contain a higher level of saponins than *microsperma* lentils with a brown testa. This information is useful to screen and select lentils with very

different levels of saponin and it would be interesting to conduct a breeding programme for the manipulation of the levels of these biologically active compounds making suitable crosses in order to produce varieties of lentils optimised for their end use and meeting nutritional requirements.

The use of combined HPLC-MS followed by improved purification techniques and NMR spectroscopy would be essential for the isolation and structural elucidation of possible new saponins present in legume seeds such as that found in *L. angustifolius* seed (Chapter IV).

The data in Chapter VI established the fate of soyasaponin VI in the seed matrix, which was stable after soaking and germination but unstable when chickpeas and lentils were cooked. In this sense, it would be interesting to use the improved HPLC technique described in Chapter V, which quantifies intact saponins, for a study into the effect of other kinds of traditional methods of legume processing such as fermentation or toasting and establish both the fate of the native saponins and changes in saponin content after processing. Another important effect to consider would be that of storage of both raw and processed legume seeds with its potential for bringing about changes in the chemical structure of saponins.

It has already been mentioned that several studies[185-187,259] have demonstrated a relationship between the chemical structure and biological activity of saponins. DDMP saponins have only been recently discovered and, apart from their oxygen radical scavenging properties, there is a lack of information on other properties that this particular type of saponins might have. Therefore, it would be crucial to carry out fresh biological studies in order to determine if these saponins share the properties ascribed to saponins lacking the DDMP group. For instance, since one of the better-known biological effects of conventional saponins is their capacity to permeabilise the small intestinal mucosal epithelium (Section 1.2.9.1), it would be interesting to assay the ability of either isolated saponins or extracts from raw and processed legumes, with known levels of different saponins, to influence gut transport.

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